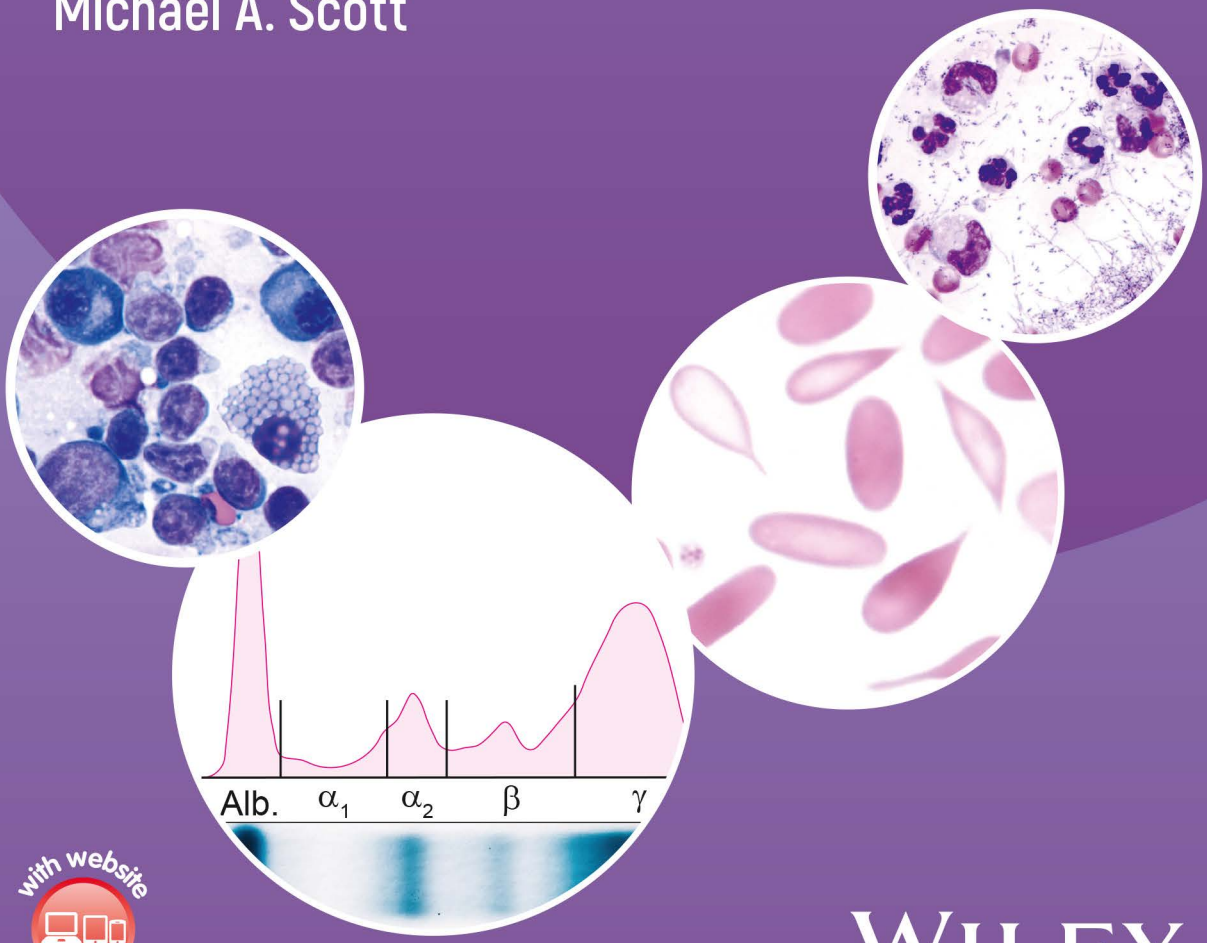
Third Edition

Fundamentals of Veterinary Clinical Pathology

Edited by
Steven L. Stockham
Michael A. Scott



with website

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Fundamentals of

VETERINARY CLINICAL PATHOLOGY

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This edition is dedicated to Dr. Steve Stockham, consummate teacher and life-long learner.

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PREFACE

Although few core principles and fundamentals of veterinary clinical pathology have changed since the second edition, a third edition was long overdue given progress in the field and the wealth of clinically relevant new information that has been generated by veterinary colleagues and others. There were new topics to address, old topics to expand on, and additional recognized disorders to consider when interpreting laboratory abnormalities. Also, a new edition provided the opportunity to improve figures and other book features.

However, the basic "blueprint" and goals of the book have not changed. Content is still focused on (i) the fundamental principles of laboratory testing, (ii) the clinical laboratory assays used to detect, define, or evaluate pathologic states in domestic animals, (iii) the pathologic, physiologic, and pharmacologic states to consider when results of clinical laboratory assays are abnormal, (iv) the pathologic, physiologic, pharmacologic, or procedural processes and mechanisms that result in abnormal laboratory data, (v) the physiologic information needed to recognize and understand abnormal states, and (vi) microscopic identification of the cells, organisms, and other structures in clinical samples that indicate or suggest the presence of common pathologic states. Content is again provided in a mixed outline/text format that includes separate sections for physiologic concepts, analytical concepts, and interpretation of abnormal results. Illustrative figures and captions complement this information, as do tables that are aligned with the text and organized into mechanistic categories.

What's new? This edition brings new co-author perspectives and expertise to 7 of the previous 19 chapters and to 1 of 2 new chapters. The new chapters focus on synovial and cerebrospinal fluids, thus completing the package of body fluid analysis. Alpacas and llamas are now included throughout the book in addition to dogs, cats, cattle, and horses. Other species (e.g., small ruminants, pigs, ferrets) are discussed only for exemplary conditions of clinical significance. Figures are now in color, molecular structures are provided where they may promote understanding, and photomicrographs are embedded where relevant to the text rather than provided in a single section of plates. Information about previous topics is updated throughout the book, and additional laboratory tests and disorders are included in nearly every chapter.

Examples of new or substantially expanded topics include analytical and biological variation, a detailed approach to blood smear evaluation, reticulocytosis without anemia, blood typing and crossmatching, viscoelastic testing, plateletcrit, positive acute-phase proteins, agarose gel and capillary zone electrophoresis, mixed acid-base disorders, and analysis of vitamin D, growth hormone, and insulin-like growth factors. References have been updated and expanded to document or provide more reading for facts or concepts that are (i) likely not widely known, (ii) particularly significant, (iii) controversial, or (iv) related to specific data, and also to provide further reading for certain topics within or beyond the scope of the book. Many excellent older references are not provided but can be found cited in many of the more recently published references.

Assimilation and synthesis of information for this edition was very educational and useful for the authors. We hope that the product of our efforts is similarly useful for other teachers of veterinary clinical pathology and for the students and veterinarians who are learning this discipline or applying it to clinical practice.

ACKNOWLEDGMENTS

FIRST EDITION

Many people directly and indirectly assisted in the writing of this book. We thank our fantastic families for providing the never-ending support for our professional pursuits. We thank our excellent veterinary and graduate students for their active participation in their learning – their questions frequently provided stimuli for finding better explanations, and their answers provided insight into what they were or were not learning. We thank our mentors (Drs. Jan Krehbiel, Doug Weiss, Harold Tvedten, and Julia Stickle) and co-residents at Michigan State University, who provided excellent specialty training. We thank Dr. Jenni Donald, who actively participated in the planning, reviewing, and critiquing of chapters. We also thank the technologists in the Clinical Pathology Laboratory at the University of Missouri, and especially Kathy Curtis, MT (ASCP), for helping us pursue many unanswered questions and for documenting certain abnormalities. We also appreciate the assistance of Howard Wilson and Don Connors during the generation of the plates and figures.

The senior author would not have accepted the challenge of writing this text without the persistent encouragement of a longtime friend, colleague, and excellent teacher—Donald A. Schmidt, DVM, PhD, Diplomate, ACVP, Professor Emeritus. Very few people have the opportunity to work with such a great person who was also one of the pioneers in the specialty of veterinary clinical pathology. His statement that "students must come first, they are the reason we are here" provided the justification for many career decisions. For over 25 years at the University of Missouri, veterinary students (and this senior author) were extremely fortunate to have Dr. Schmidt as a teacher.

The specialty in veterinary clinical pathology grew out of the increased use of laboratory tests in veterinary medicine, was promoted in the early 1960s by a society now known as the American Society for Veterinary Clinical Pathology (ASVCP), and then formalized in the early 1970s by the American College of Veterinary Pathologists (ACVP). The authors extend thanks to the ASVCP and ACVP members (and especially charter members and contemporary colleagues) for their leadership, investigations, and instruction. We have made great advances in our understanding of diseases of domestic mammals in the past 40 years, but there are many facts we do not know and processes we do not understand. The clinical laboratories, their dedicated personnel, and their expanding analytical procedures will play a key role in future advances.

SECOND EDITION

We are still greatly appreciative of the many people who directly or indirectly assisted in the writing of the first edition, and we extend sincere thanks to colleagues, students, and family who supported our efforts to produce the second edition. We appreciate the faculty in our departments and colleges who value the scholarly activities of teaching and the writing of a textbook, and we are especially thankful for the unwavering support of our spouses and for the encouraging feedback offered by veterinary students, residents, and fellow clinical pathologists.

THIRD EDITION

The authors sincerely thank all those previously acknowledged for their impact on the first two editions of this book, without which this edition would not exist. This edition would also not exist without the incredible sustained support of spouses and the work accommodations provided by

colleagues. Thank you! We also thank Mal Hoover (medical illustrator now retired from Kansas State University) for her assistance with figures, residents who provided feedback on chapter drafts, and the many veterinary students, residents, clinicians, and colleagues who stimulated us to dig deeper for more answers and better ways to share them.

Sadly, the original architect of this project – Dr. Steve Stockham – was unable to see the third edition to completion (see In Memoriam, 2020, *Vet Clin Pathol*, doi: 10.1111/vcp.12847). However, Steve helped revise several chapters for this edition, provided considerable input for all of them, and led the project even after retirement and while terminally ill with cancer. It was a privilege to work with him and directly experience the attributes that earned him numerous teaching awards, praise for his mentorship, and a Lifetime Achievement Award from the American Society for Veterinary Clinical Pathology. Steve set goals, maintained focus, worked tirelessly, communicated promptly and thoroughly, welcomed new ideas and knowledge, encouraged others to succeed, challenged others to grow, provided support, and celebrated successes. He understood and prioritized the needs of learners, and he tried to keep the content applicable, accurate, and as digestible as possible. Although every chapter has been updated since Steve's untimely death, his approach and contributions are prominent on nearly every page. We remember Dr. Stockham for his fantastic mentorship, his amazing passion and effort to teach veterinary clinical pathology, and his drive to learn, apply, and share effective teaching practices.

Thank you, Steve!

ABOUT THE COMPANION WEBSITE

This book is accompanied by a companion website:

https://www.wiley.com/go/fundamentals/vet



This website includes:

Chapter wise References

Chapter 1

ANALYTICAL AND DIAGNOSTIC CONCEPTS: GETTING IT RIGHT

Kristen R. Friedrichs, Michael A. Scott, and Steven L. Stockham

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Table 1.1. Abbreviations and symbols in this chapter	
$[fT_4]_{ed}$	Free thyroxine concentration by equilibrium dialysis
[x]	x concentration (x = analyte)
BHB	β-Hydroxybutyrate
Ca^{2+}	Calcium
CBC	Complete blood count
CLSI	Clinical Laboratory Standards Institute
CV	Coefficient of variation
CV_A	Analytical variation
CV_G	Inter-individual (between-subject) biological variation
CV_I	Intra-individual (within-subject) biological variation
EDTA	Ethylenediaminetetraacetic acid
fCa^{2+}	Free ionized calcium
FN	False negative
FP	False positive
fT_4	Free thyroxine
II	Index of individuality
K_2EDTA	Dipotassium salt of ethylenediaminetetraacetic acid
K_3EDTA	Tripotassium salt of ethylenediaminetetraacetic acid
LRL	Lower reference limit
Na_2EDTA	Disodium salt of ethylenediaminetetraacetic acid
NIST	National Institute of Standards and Technology
NPV	Negative predictive value (predictive value of a negative test)
PPV	Positive predictive value (predictive value of a positive test)
QA	Quality assurance
QC	Quality control
QCM	Quality control material
RBC	Red blood cell, erythrocyte
RCV	Reference change value
RI	Reference interval
ROC	Receiver operating characteristic
SD	Standard deviation
SI	Système international d'unités (International System of Units)
tCa^{2+}	Total calcium
TE_a	Total error allowable (allowable total error)
TE_o	Total error observed (observed total error)
TN	True negative
TP	True positive
tT_4	Total thyroxine
U	International unit
URL	Upper reference limit
USD	Usual standard deviation
WBC	White blood cell, leukocyte
WRI	Within reference interval

Note: See Table 1.4 for abbreviations of units of measurement and the figure legends for abbreviations unique to figures.

CLINICAL PATHOLOGY

I. What is clinical pathology?
   A. Definitions
      1. *Pathology* is the study of disease. It deals with understanding the structural and functional changes causing or caused by disease.
      2. *Clinical pathology*, a subtype of pathology, is the study of disease in the clinical environment by use of laboratory assays. It deals with the use of laboratory testing (clinical chemistry, hematology, …) for the diagnosis and management of disease.
   B. As currently certified by the American College of Veterinary Pathologists, veterinary clinical pathologists are specialists in the disciplines of basic pathology, hematology (study of blood and bone marrow, including hemostasis), clinical chemistry (study of physiologic and biochemical reactions in blood or other body fluids), cytologic pathology (study of cells in tissues or body fluids), surgical pathology (study of disease via microscopic analysis of tissue samples obtained during surgery), and principles of laboratory medicine (equipment and methods, test properties, QA).
   C. Veterinary clinical pathologists, medical laboratory scientists (medical technologists, clinical laboratory scientists), medical laboratory technicians, and veterinary technicians often work in a clinical laboratory that limits its assay "menu" (offered tests) to hematologic assays including hemostasis tests, clinical chemical assays, urinalysis, and cytologic examinations. Other assays or diagnostic laboratory procedures may be offered but are often provided by other specific laboratories (e.g., microbiology, histopathology, and toxicology) that are commonly supervised by microbiologists, histopathologists, and toxicologists, respectively.

II. Laboratory tests should be used purposefully, judiciously, and along with other diagnostic procedures. Before laboratory tests are used to pursue a possible diagnosis, two diagnostic procedures are imperative: (i) obtain a complete history and (ii) perform a complete physical examination. With knowledge gained from these two basic procedures, a diagnostician can select appropriate diagnostic procedures to clarify or classify identified problems prior to developing or refining a differential diagnosis and pursuing further (or definitive) testing. Veterinarians frequently use laboratory assays in conjunction with other diagnostic methods to identify or classify pathologic states that develop in domestic mammals. Some body systems (e.g., integument, nervous, skeletal, and cardiovascular) are relatively easily evaluated via visual or imaging methods (physical examination, radiography, and ultrasonography), whereas other body systems (e.g., hemic, immune, urinary, and endocrine) are better evaluated by laboratory tests. Consideration of the following questions and associated material in this chapter and elsewhere[1] will help optimize selection of cost-effective tests that benefit patient management:
   A. Why is the test being ordered? Is it needed? How will the test results influence patient management or outcome?
   B. How well does the test discriminate between health and disease? How well does it discriminate among various disorders?
   C. How are the test results interpreted? Are appropriate reference values available?

III. What are the major reasons for analyzing patient samples via laboratory procedures?
   A. To detect an unidentified pathologic state
   B. To define, classify, or confirm a pathophysiologic disorder or disease state
   C. To eliminate a possible cause of the animal's illness
   D. To assess changes in a pathologic state either due to natural progression of the disease or because of medical or surgical intervention

SAMPLES

I. Whole blood, plasma, and serum
   A. Most clinical laboratory assays are designed to detect or quantify substances or cells in blood samples; the substance or cell of interest is called the *analyte* while the quantity that is measured is called the *measurand*. Obtaining useful results for the analyte requires appropriate samples. Whenever there is doubt about the appropriate sample for a particular test at a particular laboratory, the laboratory should be contacted prior to sample collection. (See Quality of Laboratory Results, Section I.B.)
   B. Whole blood (blood)
      1. Blood and its major components are frequently used as samples for laboratory assays. Blood must be collected and processed properly so that assay results reflect the true composition of blood rather than artifactual changes.
      2. Blood is composed of cells (RBCs, platelets, and five major WBC types) and plasma. Blood withdrawn from a blood vessel must immediately be mixed with an anticoagulant to prevent initiation of clot formation and to maintain cells and other components in suspension.
      3. Analysis or processing of whole blood must be relatively rapid because the cells deteriorate or die within a few hours, and thus a sample becomes unacceptable for analysis. What constitutes adequate sample handling varies with what is to be quantified or evaluated; occasionally, samples must be analyzed within minutes, usually within hours, rarely within days.
   C. Plasma
      1. *Plasma* is the fluid component of blood that is harvested after centrifugation of an anticoagulated blood sample. In vitro, plasma contains an anticoagulant that can interfere with some assays. Unlike serum, plasma contains fibrinogen.
      2. Anticoagulants used for blood sample collection
         a. Calcium-binding agents prevent Ca^{2+} from participating in the formation of a blood clot.
            (1) EDTA (as K_3EDTA, K_2EDTA, or Na_2EDTA)
               (a) EDTA is the preferred anticoagulant for almost all routine hematologic tests, including the complete blood count (CBC) assays.
               (b) EDTA chelates Ca^{2+} and other divalent cations (Mg^{2+}, Cu^{2+}, and Pb^{2+}) but the other anticoagulants do not. A *chelating agent* forms at least two bonds with the metallic ion it chelates. EDTA attaches to Ca^{2+} in six places.
               (c) Blood and anticoagulant should be mixed promptly with at least eight gentle and complete inversions of the tube; one inversion is defined as tipping the tube 180° and back again.
            (2) Citrate (as sodium citrate)
               (a) Citrate is the preferred anticoagulant for most tests of hemostasis. During coagulation tests, a specific amount of Ca^{2+} is added to the citrated plasma to override the effects of citrate and enable coagulation enzymes to function. Citrate's anticoagulant activity is achieved by its formation of a single ionic bond with Ca^{2+}.
               (b) Because it has low toxicity, citrate is also preferred for collection of whole blood to be used for transfusions.
               (c) Blood and anticoagulant should be mixed promptly with at least four gentle and complete inversions of the tube; one inversion is defined as tipping the tube 180° and back again.

(3) Oxalates (potassium salt is the most common)
(a) Oxalate is used for a few laboratory tests. It is the anticoagulant in sodium fluoride tubes that can be used for glucose and lactate assays. Generally, oxalates distort morphologic features of WBCs and RBCs and thus are unsuitable for hematologic samples.
(b) Oxalate's anticoagulant activity is achieved by its formation of an ionic bond with Ca^{2+}.

b. Heparin (as lithium, ammonium, potassium, or sodium salts) activates antithrombin (also called antithrombin III), which then inhibits the activity of coagulation enzymes, including thrombin. It also forms an ionic bond with Ca^{2+}, but its major action is through antithrombin.
(1) Heparin is used for several special laboratory assays (such as blood gas analysis) and for many clinical chemistry assays.
(2) Disadvantages
(a) May cause pink staining of cells and extracellular regions of blood smears, but it is commonly used for CBC samples from many nonmammalian species
(b) Allows minor clotting to occur over time as effects are slowly overridden by the coagulation system
(c) Allows or promotes platelet (thrombocyte) clumps to form
(3) Blood and anticoagulant should be mixed promptly with at least eight gentle and complete inversions of the tube; one inversion is defined as tipping the tube 180° and back again.

c. Some anticoagulant tubes contain a polymer gel that begins in the bottom of the tube but re-forms at the cell-plasma interface with centrifugation. This creates a barrier to decrease cell effects on plasma, facilitate pipetting plasma without cells, and increase plasma yield.

3. Plasma has two major components.
a. Water: about 92–95 % of plasma volume; 100 mL of plasma contains 92–95 mL of H_2O.
b. Solids: about 5–8 % of plasma volume; most solids are proteins on a weight per volume (weight/volume) basis. Other solids are glucose, urea, electrolytes, and other chemicals.

4. Generally, the chemical composition of plasma is very similar to interstitial fluid in most tissues. Within the body, plasma and interstitial fluid are the extracellular fluids, intravascular and extravascular, respectively.

D. Serum
1. Serum is the fluid component of blood that is harvested after centrifugation of a coagulated (clotted) blood sample in a blood tube commonly referred to as a clot tube. As described in Chapter 5, blood clotting involves platelets and coagulation proteins. To get the maximal amount of serum from a clotted sample, centrifugation should not be started prior to the retraction of the clot; this typically takes at least 30 min (45 min for equine samples) if a clot activator is not present in the tube. If samples are centrifuged prior to clot retraction, a soft fibrin clot resembling a veil may extend into the serum layer and reduce the serum yield; this is most often seen with equine samples.
2. Serum has essentially the same composition as plasma except serum does not contain most of the coagulation proteins. The major protein (on a weight/volume basis) that is absent in serum but present in plasma is fibrinogen. Fibrinogen is the only protein that contributes significantly to differences in paired serum and plasma total protein

concentrations, but such differences often occur for other reasons, including use of different methods (refractometry for plasma versus chemical methods for serum).

3. During the clotting process, substances released from cells may alter analyte concentrations in serum. For example, platelets release K^+, and thus serum [K^+] is greater than plasma [K^+] (see Chapter 9).
4. If the tube contains a clot activator, the tube should be gently inverted five times; one inversion is defined as tipping the tube 180° and back again.
5. Some clot tubes contain a polymer gel that begins in the bottom of the tube but re-forms at the cell-plasma interface with centrifugation. This creates a barrier to decrease ongoing cell effects on serum, facilitate pipetting serum without cells, and increase serum yield.

E. Order of blood sample collection for routine tubes: Blood samples are generally collected in the following order to avoid cross-contamination of tube additives and subsequent preanalytical error in results, but there can be reasons to alter the order for specific situations. Consider similar factors if collecting blood into other tube types.

1. Blood culture tube or bottle: to prevent contamination with potentially growth-inhibiting additives in other tubes
2. Sodium citrate tube (light blue cap): collected early to avoid (i) contamination by anticoagulants or clot activators from other blood tubes and (ii) activation of coagulation prior to sample anticoagulation
3. Serum tubes, including those with clot activator and gel (red, gold, or mottled caps): to avoid contamination with anticoagulants that may alter results (e.g., contaminating K_3EDTA may falsely increase [K^+] and falsely decrease [tCa^{2+}])
4. Sodium or lithium heparin tube, with or without gel (green, light green, or speckled green cap): to avoid contamination with EDTA that may alter results as noted for serum
5. EDTA tube (lavender or pink cap)

II. Urine samples

A. Other than blood, urine is the most common sample analyzed by laboratory assays. As with blood, urine must be collected and processed properly so that the assay results reflect the true composition of the product of the urinary system.

B. To prevent artifactual changes in urine, it should be processed soon after collection. General guidelines for the collection and processing of urine for routine analyses are described in Chapter 8.

III. Other body fluid samples

A. Pleural fluid, peritoneal fluid, pericardial fluid, synovial fluid, and cerebrospinal fluid samples are collected to characterize body cavity effusions, joint diseases, and central nervous system disorders, respectively.

B. The processing and handling of cavitary fluids are described in Chapter 19, and synovial fluid and cerebrospinal fluid analyses are described in Chapters 20 and 21, respectively.

IV. Nonfluid samples for cytologic or histologic evaluation

A. Air-dried cytologic preparations can be obtained from any tissue in the body to characterize various processes and lesions. Cells are collected by needle, scraping, swabbing, or imprinting, depending on the nature of the lesion. Such sampling of lymph nodes and bone marrow is described in Chapter 6.

B. Antemortem histologic samples of lesions are collected by various incisional or excisional techniques followed by fixation and processing for sectioning. Collection of bone marrow histologic samples (cores) is described in Chapter 6.

COMMON TYPES OF CLINICAL PATHOLOGY LABORATORY ASSAYS

I. Many laboratory tests or assays involve the analysis of body fluids (whole blood, serum, plasma, urine, peritoneal fluid, pleural fluid, pericardial fluid, cerebrospinal fluid, and synovial fluid), tissue samples, or feces. Most clinical laboratory procedures fall into one of the three large groups below (examples follow subdivisions); many procedures could be classified into more than one group.
   A. Clinical hematology assays: Most assays are completed on whole blood samples.
      1. Quantitation of cell concentrations in blood: total WBC concentration (count), [RBC], and [platelet]
      2. Semiquantitation of cell concentrations: calculated [WBC] and platelet estimate from blood film examination
      3. Defining or classifying cells by microscopic features: toxic neutrophils, reactive lymphocytes, polychromatophilic RBCs, poikilocytes, microcytes, hypochromic RBCs, and leukemic cells
      4. Assessing the hemostatic properties of blood: clotting times and platelet function assays
      5. Assessing hematopoiesis and bone marrow health: bone marrow aspirate and core biopsies
   B. Clinical chemistry assays: Most assays are completed on serum or plasma samples.
      1. Detecting or quantifying the concentration of a chemical substance
         a. Quantitative analysis: Results are close to the true concentration (e.g., serum concentrations of glucose, sodium, protein, creatinine, and urea) and typically are reported with a specific numerical value (see the Significant Digits section).
         b. Semiquantitative analysis: Results are "within the ballpark" (e.g., urine glucose, protein, and bilirubin concentrations by reagent pad chemistry assays) and may be reported as approximate numerical values or in a categorical scale (e.g., 1+, 2+, or 3+) that represents ranges of numerical values.
         c. Qualitative analysis: Results indicate that a substance is or is not present (e.g., fat detected microscopically in pleural fluid with a Sudan stain) and are reported as "present" or "absent" or as "positive" or "negative."
      2. Detecting or quantifying the activity of a chemical substance
         a. Quantitative analysis: Results are close to true activity (e.g., measured activities of serum enzymes such as alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, and creatine kinase).
         b. Qualitative analysis: Results indicate that activity is or is not present (e.g., heme's peroxidase activity or leukocyte esterase activity in urine).
   C. Clinical microscopy
      1. Clinical cytology: the study of cell populations and their microscopic features in an attempt to define or classify abnormal tissue or fluid (e.g., fine-needle biopsy smears from lymph nodes to diagnose lymphoma or histoplasmosis, fine-needle biopsy smears of a skin mass to determine whether it is an inflammatory or neoplastic lesion, and analysis of peritoneal fluid to determine if it is a transudate, exudate, or other type of effusion)
      2. Surgical histopathology: the study of frozen or fixed tissue to define or classify abnormal tissue (i.e., inflammatory, neoplastic, and toxic disorders) and perhaps establish an etiologic diagnosis
      3. Urine sediment analysis: microscopic examination of wet or air-dried urine preparations to detect or semiquantify the presence of WBCs, RBCs, casts, bacteria, crystals, or other structures
      4. Clinical parasitology: microscopic analysis of fecal, urine, blood, or other samples to detect ova, larvae, or other microscopic forms of parasites
      5. Clinical hematology: noted above in Section I.A

II. Actual descriptions of the numerous laboratory methods are beyond the scope of this textbook. However, an understanding of basic principles and methods is frequently needed to interpret results of a laboratory assay. Such principles are located in several parts of the textbook, and specific methods can be provided by laboratory personnel or manuals.
   A. Chapters 2–5 contain the basic principles and concepts of the common hematologic assays.
   B. Chapter 6 contains the guiding principles and overview of the analysis of bone marrow and lymph nodes, the two major tissues involved in hematopoiesis.
   C. Chapters 7–18 have short sections that describe analytical principles that apply to other individual analytes.
   D. Chapters 19–21 contain basic principles pertaining to the analysis of cavitary effusions, synovial fluid, and cerebrospinal fluid.

SIGNIFICANT DIGITS (SIGNIFICANT FIGURES)

I. Results of many laboratory assays consist of a number, often calculated, accompanied by a unit of measure and RIs. Although many reporting systems do not allow it, the number should be reported to the appropriate number of digits by following three basic rules for reporting significant digits.
   A. Retain only as many significant digits in a result as will give only one uncertain digit (Table 1.2). A digit is uncertain when the associated measurement cannot reliably

Table 1.2. Examples of significant digits

Number	Significant digits	Reason for the given number of significant digits
124	3	All digits imply significance.
124.0	4	The zero was not needed for the whole number. Its addition indicates that it is a significant digit.
120	2 or 3	If the zero is just preserving space, it is not a significant digit. If values are usually reported to the ones place in a particular setting (e.g., 121), then the zero is a significant digit.
120.0	4	The zero in the tenths space indicates it is a significant digit. Thus, the zero in the ones place is also a significant digit.
0.12	2	The zero is only preserving a space and is not a significant digit. It is often written by convention such that the "." is recognized as a decimal point and not a period, fly dirt, or other extraneous material.
0.02	1	The zero in the tenths place is just filling space and is not a significant digit.
0.020	2	The zero in the thousandths place indicates it is significant.

Note: The number of significant digits is determined by starting with the left-most nonzero digit and counting until reaching the last significant digit. If numbers are properly reported, nonzero digits are always significant, as are all zeros between other significant digits and any zeros that are to the right of the decimal point and terminal. If no decimal point is present, any terminal zeros after the right-most nonzero digit may or may not be significant (such as those in 17,000).

differentiate the value in that digit from one above or one below it. Zero is not a significant digit if it only preserves a space in the number.

B. When reporting results from multiplications or divisions that use two or more numbers with appropriate significant digits, the final calculated result should have no more significant digits than the number(s) with the fewest significant digits.
   1. For $1.23 \times 2.4 = 2.952$, the product should be reported as 3.0 because 2.4 has only two significant digits.
   2. Multiplying 0.03 and 0.12, which have one and two significant digits, respectively, yields 0.0036, but this should be reported to one significant digit: 0.004.
   3. If MCV = 71 fL and [RBC] = 3.8×10^9/L, the analyzer's Hct = $71 \times 3.8 / 10 = 26.98$ %, which is 27 % when reported appropriately to two significant digits.

C. When reporting results from additions or subtractions that use two or more numbers with appropriate significant digits, the final calculated result should have no more decimal places than the number(s) with the fewest decimal places. For $1.23 + 2.4 = 3.63$, the sum should be reported as 3.6.

II. As in the previous examples, it is frequently necessary to round numbers so that only significant digits are reported. There are various rules for rounding numbers when the last digit is 5, some being to round up the preceding digit whether it is even or odd. However, other rules are used to remove upward or downward rounding bias in data sets, as follows.

A. Add 1 to the last retained digit if it is odd (round up, example C.1).

B. Do not add 1 to the last retained digit if it is even (round down, example C.2).

C. Examples with rounding to two significant digits: The third example (C.3) does not require the rounding rule because 4 is the digit to be rounded – not 5 – when rounding to two significant digits; scientific notation is used to clarify that the zeroes following the significant digits in 1100, 1200, and 1300 are not significant digits.
   1. $1150 \rightarrow 1.2 \times 10^3$
   2. $1250 \rightarrow 1.2 \times 10^3$ (would round up to 1.3×10^3 with some rules)
   3. $1145 \rightarrow 1.1 \times 10^3$

III. Using the rules of significant digits for reporting results is one way that laboratory personnel can communicate the known precision and measurement uncertainty of the laboratory's assay result. The first rule of significant digits (see Significant Digits, Section I.A) is to retain only one uncertain digit. The following illustrates how that uncertain digit can be determined.

A. One sample is analyzed many times (e.g., 20). The mean and SD of the data are calculated.

B. If, for a given assay, the mean value was 125.345 and the SD was 0.578, then the interval that represents the mean ± 2 SD is 124.189–126.501. With this degree of analytical precision, the assay can readily distinguish between 100 and 200 and between 120 and 130, but it may not be able to distinguish consistently between 125 and 126. Thus, the value in the ones place is uncertain and the measured value should be reported to the nearest whole number (i.e., the mean = 125).

C. If, for a second assay, the mean value was 125.345 and the SD was 9.111, then the interval that represents the mean ± 2 SD is 107.123–143.567. With this degree of analytical precision, the assay can readily distinguish between 100 and 200, but it probably cannot reliably distinguish between 120 and 130. Thus, the tens value is uncertain, and the measured value should be reported to the nearest tens unit, making the mean value 120 or 130, depending on the rounding rule used for 5s (see preceding section).

D. Understanding significant digits becomes important when laboratory data are reported and interpreted. If one understands that we are uncertain about the last reported significant digit, then it is less likely that a 120 in today's sample and a 130 in tomorrow's sample will be considered a true biological change. Unfortunately, there are several reasons that all results are not reported this way:
   1. It may not be the policy for some laboratories.
   2. Analyzers and data management programs may not follow rules of significant digits and may not be modifiable.
   3. The appropriate number of places after the decimal point may vary for a particular analyte, depending on the magnitude of the value, but analyzers and software may not allow such variability. For example, precision is usually worse at low concentrations, so the appropriate number of significant digits may be greater at higher concentrations. However, for any analyte, most laboratories report all values to the same apparent degree of precision relative to the decimal point. This may lead to overestimation of the precision of smaller measurements or underestimation of the precision of larger measurements. An example is shown in Figure 1.1.

Measured values
WBC concentration = 21.5 × 10^9/L
WBC diff. count: 73 % neutrophils, 12 % lymphocytes, 9 % monocytes, 4 % eosinophils, 2 % basophils

Calculations and resultant significant figures
Neutrophils: (**21.5** × 10^9/L) × 0.**73** = 15.695 × 10^9/L = **16** × 10^9/L
Lymphocytes: (**21.5** × 10^9/L) × 0.**12** = 2.58 × 10^9/L = **2.6** × 10^9/L
For these results: 3 *significant figures* × 2 *significant figures* = 2 *significant figures*

Monocytes: (**21.5** × 10^9/L) × 0.0**9** = 1.935 × 10^9/L = **2** × 10^9/L
Eosinophils: (**21.5** × 10^9/L) × 0.0**4** = 0.86 × 10^9/L = 0.**9** × 10^9/L
Basophils: (**21.5** × 10^9/L) × 0.0**2** = 0.43 × 10^9/L = 0.**4** × 10^9/L
For these results: 3 *significant figures* × 1 *significant figure* = 1 *significant figure*

However, most computer-based reporting systems require reporting analytes' values consistently to a defined decimal point and thus significant figure rules will not be consistently applied.

	Results using significant figure rules	Results using consistent decimal points
Total WBC	21.5 × 10^9/L	21.5 × 10^9/L
Neutrophils	16 × 10^9/L	16.0 × 10^9/L
Lymphocytes	2.6 × 10^9/L	2.6 × 10^9/L
Monocytes	2 × 10^9/L	2.0 × 10^9/L
Eosinphils	0.9 × 10^9/L	0.9 × 10^9/L
Basophils	0.4 × 10^9/L	0.4 × 10^9/L

Figure 1.1. Significant digits. The significant digits of the measured and calculated values of a leukogram are provided. When two values are multiplied, the result should have no more significant digits than the number with the fewest significant digits (see I.B in this section). However, computer-based reporting systems may not enable the flexibility to report only significant digits.
diff., differential

UNITS

I. SI units versus nonSI (conventional) units

A. For several decades, there has been an attempt to switch to a metric system of units throughout the world. Other than the United States of America, the conversion is mostly complete in a modified practical form. During the 1970s and 1980s, several organizations in the United States attempted to convert members of the medical communities to SI units but had limited success. Because there is a lack of consistent use of the SI unit system, veterinary medical professionals need to be familiar with both SI and nonSI units.

B. In the context of units used for laboratory data, the basic units of measurement are listed in Table 1.3. Many clinical laboratory and professional organizations have agreed to use liter as the preferred unit for volume instead of the SI unit of cubic meter because the latter is rarely clinically relevant (1 m^3 = 1000 L). Even the use of liter for a volume unit has limited relevance in the clinical laboratory when the sample volume for many assays is less than 0.1 mL.

C. Because the reported units for amount or concentration (a concentration is an amount per volume) of substances vary considerably, a veterinary medical professional should know the common abbreviations for the major units (Table 1.4). A common error is to translate dL to decaliter (10 L) rather than deciliter (0.1 L).

D. The National Institute of Standards and Technology (NIST) provides rules and style conventions for the use of the SI units to reduce ambiguity in scientific communications. Examples of the NIST unit conventions used in this book are in Table 1.5.

E. Table 1.6 contains the formulas for the conversion of analyte values from nonSI units to SI units; only analytes presented in this textbook are included. The table contains two types of formulas: (i) formulas that show the simple conversion factor that is used to calculate the numerical value of the SI unit,[2] and (ii) formulas that show the conversion of the numerical value and units.

Table 1.3. Examples of measurement in SI units and conventional units

	SI units	Conventional units
Amount of substance	mole (mol)	gram (g)
Length	meter (m)	yard, foot, inch
Mass	kilogram (kg)	pound (lb), grain
Time	second (s)	minute (min), hour (h)
Volume	cubic meter (m^3)	liter (L)

Table 1.4. Common units and abbreviations for laboratory values

Number[a]		Volume		Mass	
—	—	—	—	kg	kilogram, 10^3 g
mol	mole	L	liter	g	gram
—	—	dL	deciliter, 10^{-1} L	—	—
mmol	millimole, 10^{-3} mol	mL	milliliter, 10^{-3} L	mg	milligram, 10^{-3} g
µmol	micromole, 10^{-6} mol	µL	microliter, 10^{-6} L	µg	microgram, 10^{-6} g
nmol	nanomole, 10^{-9} mol	nL	nanoliter, 10^{-9} L	ng	nanogram, 10^{-9} g
pmol	picomole, 10^{-12} mol	pL	picoliter, 10^{-12} L	pg	picogram, 10^{-12} g
fmol	femtomole, 10^{-15} mol	fL	femtoliter, 10^{-15} L	fg	femtogram, 10^{-15} g

[a] Based on moles, 1 mole being Avogadro's number ($\sim 6.022 \times 10^{23}$) of particles of a substance

Table 1.5. Examples of NIST style for writing units compared to other styles[a]

Rules	NIST	NonNIST
Symbols unaltered in the plural	20 pg 4 h	20 pgs 4 hrs
Symbols not followed by period unless end of sentence	2 L 6 yr	2 L. 6 yr.
There is a space between the number and the unit	10 % 37 °C 15 g/dL	10% 37°C, 37° C 15g/dL
No words are inserted between the number and the unit	3000/µL	3000 WBC/µL

[a] The NIST system has two styles for a range of numbers, e.g., 5 % to 10 % or (5 to 10) %. To save space and to conform to common use in veterinary medicine, the numbers are displayed as follows in this textbook: 5–10 %.

II. Amount versus concentration: One important basic concept for interpreting laboratory data is having a clear understanding of what a laboratory test result represents. Besides knowing what is really being measured, it is important to understand what the numbers and units represent. A number without a unit is meaningless. The following examples illustrate the concepts:
   A. A dog acutely lost a large amount of blood because of an injury. Because whole blood including RBCs was lost, the number of RBCs in the body is decreased. However, because plasma was lost with the RBCs, the [RBC] (number of RBCs per volume of blood) in the dog initially is not decreased, and thus the dog is not initially anemic. After fluid shifts restore plasma volume, the dog will have fewer RBCs in its body and a lower [RBC] in its blood.
   B. You are told that a cat's serum sodium level was increased. Does this mean the cat has more sodium in its body? Well, it might. However, the increased serum [Na^+] might be due to less water in the body, and the amount of sodium may not be increased. In fact, the total amount of sodium in the body could be decreased if there was relatively more water loss than sodium loss.
   C. You are told that a horse's serum enzyme level was decreased. Does this mean the horse has less of that enzyme? It might. However, it could be that the amount of enzyme (the protein) was not decreased, but the enzyme's activity was inhibited, or the structure of the enzyme was defective.
   D. You are told that a cat's reticulocyte percentage is increased. Does this mean that the cat has more reticulocytes in its blood? It might or might not. A percentage is always relative; the same number in the numerator (e.g., number of reticulocytes counted) and a smaller number in the denominator (e.g., total number of RBCs counted) results in an increased percentage, so there might be fewer RBCs rather than more reticulocytes.
   E. You are told that the myeloid to erythroid ratio in a cat's marrow is increased. Does the increased ratio mean the cat's marrow contains more myeloid cells, fewer erythroid cells, or both? Or is the ratio increased because there are more myeloid and more erythroid cells, but the number of myeloid cells is increased more than the increase in erythroid cells? A calculated ratio is always a relative number and must be interpreted accordingly.
   F. You are told that a dog's urine has an increased [protein]. Because the concentrations of all substances in urine depend on the conservation of water by the kidneys, the increased [protein] could have resulted from increased water conservation and not increased protein loss via the urinary system.

Table 1.6. Conversion of nonSI units to SI units

Analyte[a]	To convert		Multiply by[b]	Complete conversion formulas	Increment[c]
	From	To			
Acetoacetate	mg/dL	µmol/L	99	mg/dL × 9.9 µmol/mg × 10 dL/L = µmol/L	—
ACTH	pg/mL	pmol/L	0.2202	pg/mL ÷ 4541 pg/pmol × 1000 mL/L = nmol/L	1 pmol/L
Albumin	g/dL	g/L	10	g/dL × 10 dL/L = g/L	1 g/L
Aldosterone	ng/dL	pmol/L	27.74	µg/dL ÷ 360.5 µg/µmol × 1000 nmol/µmol × 10 dL/L = nmol/L	10 pmol/L
β-Hydroxybutyrate	mg/dL	µmol/L	97	mg/dL × 9.7 µmol/mg × 10 dL/L = µmol/L	—
Bile acid (total)	mg/L	µmol/L	2.547	mg/L ÷ 392.6 µg/µmol × 1000 µg/mg = µmol/L	0.2 µmol/L
Bile acid (total)	mg/mL	mmol/L	2.547	mg/mL ÷ 392.6 mg/mmol × 1000 mL/L = mmol/L	0.2 mmol/L
Bt	mg/dL	µmol/L	17.10	mg/dL ÷ 584.8 mg/mmol × 1000 µmol/mmol × 10 dL/L = µmol/L	2 µmol/L
Cholesterol	mg/dL	mmol/L	0.02586	mg/dL ÷ 386.7 mg/mmol × 10 dL/L = mmol/L	0.05 mmol/L
Cl^-	mEq/L	mmol/L	1	mEq/L × 1 mmol/mEq = mmol/L	1 mmol/L
Cl^-	mg/dL	mmol/L	0.2817	mg/dL ÷ 35.5 mg/mmol × 10 dL/L = mmol/L	1 mmol/L
COP	mmHg	pascals	133.322	mmHg × 133.322 pascals/mmHg = pascals	—
Cortisol	µg/dL	nmol/L	27.59	µg/dL ÷ 362.45 µg/µmol × 1000 nmol/µmol × 10 dL/L = nmol/L	10 nmol/L
Creatinine	mg/dL	µmol/L	88.4	mg/dL ÷ 113.1 mg/mmol × 1000 µmol/mmol × 10 dL/L = µmol/L	10 µmol/L
Cyanocobalamin	pg/mL	pmol/L	0.7378	pg/mL ÷ 1355 pg/pmol × 1000 mL/L = pmol/L	10 pmol/L
fCa^{2+}	mg/dL	mmol/L	0.2495	mg/dL ÷ 40.08 mg/mmol × 10 dL/L = mmol/L	0.01 mmol/L
fCa^{2+}	mEq/L	mmol/L	0.5	mEq/L × 0.5 mmol/mEq = mmol/L	0.01 mmol/L
Fe	µg/dL	µmol/L	0.1791	µg/dL × 0.01791 µmol/µg × 10 dL/L = µmol/L	1 µmol/L
Ferritin	ng/mL	µg/L	1	ng/mL × 1 µg/1000 ng × 1000 mL/L = µg/L	10 µg/L
Fibrinogen	mg/dL	g/L	0.01	mg/dL × g/1000 mg × 10 dL/L = g/L	0.1 g/L
Fibrinogen	mg/dL	µmol/L	0.0294	mg/dL × 0.00294 µmol/mg × 10 dL/L = µmol/L	—
Folate	ng/mL	nmol/L	2.266	ng/mL ÷ 441.3 ng/nmol × 1000 mL/L = nmol/L	2 nmol/L
fT_4	ng/dL	pmol/L	12.87	ng/dL ÷ 777 ng/nmol × 1000 pmol/nmol × 10 dL/L = pmol/L	1 pmol/L
Globulins	g/dL	g/L	10	g/dL × 10 dL/L = g/L	1 g/L
Glucose	mg/dL	mmol/L	0.05551	mg/dL ÷ 180.1 mg/mmol × 10 dL/L = mmol/L	0.1 mmol/L

Continues

Table 1.6. Conversion of nonSI units to SI units (Continued)

Analyte[a]	To convert		Multiply by[b]	Complete conversion formulas	Increment[c]
	From	To			
HCO_3^-	mEq/L	mmol/L	1	mEq/L × 1 mmol/mEq = mmol/L	1 mmol/L
Hgb	g/dL	g/L	10	g/dL × 10 dL/L = g/L	1 g/L
IRG	pg/mL	ng/L	1	pg/mL ÷ 1000 pg/ng × 1000 mL/L = ng/L	10 ng/L
IRI	µU/mL	pmol/L	7.175	µU/mL ÷ 139.4 µU/pmol × 1000 mL/L = pmol/L	5 pmol/L
IRI	µg/L	pmol/L	172.2	µg/L ÷ 5.807 µg/µmol × 1000 pmol/µmol = pmol/L	5 pmol/L
K^+	mEq/L	mmol/L	1	mEq/L × 1 mmol/mEq = mmol/L	0.1 mmol/L
K^+	mg/dL	mmol/L	0.2564	mg/dL ÷ 39 mg/mmol × 10 dL/L = mmol/L	0.1 mmol/L
L-Lactate	mg/dL	mmol/L	0.112	mg/dL × 1.11 mmol/mg × 10 dL/L = mmol/L	—
MCHC	g/dL	g/L	10	g/dL × 10 dL/L = g/L	—
Na^+	mEq/L	mmol/L	1	mEq/L × 1 mmol/mEq = mmol/L	1 mmol/L
Na^+	mg/dL	mmol/L	0.4348	mg/dL ÷ 23 mg/mmol × 10 dL/L = mmol/L	1 mmol/L
NEFA	mEq/L	mmol/L	1	mEq/L × mmol/mEq = mmol/L	—
NH_3	µg/dL	µmol/L	0.5871	µg/dL ÷ 17.03 µg/µmol × 10 dL/L = µmol/L	5 µmol/L
NH_4^+	µg/dL	µmol/L	0.5543	µg/dL ÷ 18.04 µg/µmol × 10 dL/L = µmol/L	5 µmol/L
Pi	mg/dL	mmol/L	0.3229	mg/dL ÷ 30.97 mg/mmol × 10 dL/L = mmol/L	0.05 mmol/L
Platelet	#000/µL	# × 10^9/L	10^9	#000/µL × 10^6 µL/L = # × 10^9/L	10 × 10^9/L
RBC	# × 10^6/µL	# × 10^{12}/L	10^6	# × 10^6/µL × 10^6 µL/L = # × 10^{12}/L	—
SDMA	µg/dL	µmol/L	0.0495	µg/dL ÷ 202.25 µmol/µg × 10 dL/L = µmol/L	—
T_3	ng/dL	nmol/L	0.01536	ng/dL ÷ 651 ng/nmol × 10 dL/L = nmol/L	0.1 nmol/L
T_3	pg/dL	nmol/L	15.36	pg/dL ÷ 651 pg/pmol × 1000 pmol/nmol × 10 dL/L = nmol/L	0.1 nmol/L
tCa^{2+}	mg/dL	mmol/L	0.2495	mg/dL ÷ 40.08 mg/mmol × 10 dL/L = mmol/L	0.02 mmol/L
tCa^{2+}	mEq/L	mmol/L	0.5	mEq/L × 0.5 mmol/mEq = mmol/L	0.02 mmol/L

TIBC	µg/dL	µmol/L	0.1791	µg/dL × 0.01791 µmol/µg × 10 dL/L = µmol/L	1 µmol/L
tMg^{2+}	mg/dL	mmol/L	0.4114	mg/dL ÷ 24.31 mg/mmol × 10 dL/L = mmol/L	0.02 mmol/L
tMg^{2+}	mEq/L	mmol/L	0.5	mEq/L × 0.5 mmol/mEq = mmol/L	0.02 mmol/L
Total protein	g/dL	g/L	10	g/dL × 10 dL/L = g/L	1 g/L
Triglyceride	mg/dL	mmol/L	0.01129	mg/dL ÷ 885.7 mg/mmol × 10 dL/L = mmol/L	0.05 mmol/L
TSH	µg/dL	ng/mL	10	µg/dL × 1000 ng/µg ÷ 100 mL/dL = ng/mL	—
tT_4	µg/dL	nmol/L	12.87	µg/dL ÷ 777 µg/µmol × 1000 nmol/µmol × 10 dL/L = nmol/L	1 nmol/L
tT_4	ng/mL	nmol/L	1.287	ng/mL ÷ 777 ng/nmol × 1000 mL/L = nmol/L	1 nmol/L
UN	mg/dL UN	mmol urea/L	0.3570	mg/dL ÷ 28.01 mg/mmol × 10 dL/L = mmol of urea/L	0.5 mmol/L
Urea	mg/dL urea	mmol urea/L	0.1665	mg/dL ÷ 60.06 mg/mmol × 10 dL/L = mmol of urea/L	0.5 mmol/L
WBC	#000/µL	$\times 10^9$/L	0.001	#000/µL × 10^6 µL/L = #000 × 10^6/L = # × 10^9/L	—
Xylose	mg/dL	mmol/L	0.06661	mg/dL ÷ 150.1 mg/mmol × 10 dL/L = mmol/L	0.1 mmol/L

[a] ACTH, adrenocorticotropic hormone; Bt, total bilirubin; Cl^-, chloride; COP, colloidal osmotic pressure; Fe, iron; HCO_3^-, bicarbonate; Hgb, hemoglobin; IRG, immunoreactive glucagon; IRI, immunoreactive insulin; K^+, potassium; MCHC, mean cell hemoglobin concentration; Na^+, sodium; NEFA, nonesterified fatty acids; NH_3, ammonia; NH_4^+, ammonium; Pi, inorganic phosphorus; SDMA, symmetrical dimethylarginine; T_3, triiodothyronine; TIBC, total iron-binding capacity; tMg^{2+}, total magnesium; TSH, thyroid-stimulating hormone; and UN, urea nitrogen

[b] *Source*: Lundberg et al.[2]

[c] Recommended smallest reporting interval of the SI unit,[2] which may differ with magnitude, species, and assay precision; a reporting interval or increment of 1 g/L means that results would be reported to the nearest 1 g/dL with no decimal places to suggest less uncertainty than is actually present

ANALYTICAL AND BIOLOGICAL VARIATION

I. Appropriate use of laboratory results to monitor disease or differentiate disease from health requires an understanding of analytical variation, biological variation, and the potential contribution of various types of error to variations in results.

II. Variation in a set of data from an individual or population can be expressed as a standard deviation (SD, σ) or as a coefficient of variation (CV).
   A. SD describes the dispersion of a dataset relative to its mean and is calculated as the square root of the variance, which is the average of the squared differences of each value from the mean. The greater the SD, the more the data are spread from the mean.
   B. The CV of a set of measures is the SD reported as a percentage of the mean (Eq. 1.1). The greater the CV, the greater the variation in the data. Analytical and biological variation are commonly described in terms of CV.

$$\text{CV (expressed as a percentage)} = \frac{\text{standard deviation}}{\text{mean}} \times 100 \qquad (\mathbf{1.1})$$

III. Analytical variation is the variation in results caused by imprecision or inaccuracy of analytical methods.
   A. *Imprecision*, commonly denoted as the CV of repeated measures, is the unavoidable random error that occurs during analysis because of variations in reagent mixing, temperatures, electrical supply, operator, timing, pipetting, etc.
   B. *Bias*, or *systematic error*, is the inaccuracy of results caused by differences in instruments or methods rather than random variations and chance. When results from different methods or instruments are compared, method and instrument bias contribute to analytical variation.

IV. *Biological variation*, the inherent variation of repeated measures within a healthy individual or population, impacts many fundamental areas of laboratory medicine including method selection, quality goals (TE_a), QC testing, result reporting, and use of clinical decision limit guidelines.[3,4] There are different types.
   A. *Intra-individual (within-subject) variation*, the variation of a test result from an individual, is commonly denoted CV_I. This fluctuation around an individual's homeostatic set point occurs because of physiologic changes. Predictable physiologic variations such as circadian, reproductive, or seasonal changes are typically addressed separately by appropriate timing of sample collection and interpretation based on expected physiologic differences or a similar reference population.
   B. *Inter-individual (between-subject) variation*, the variation of test results within a population or group, is commonly denoted CV_G. This variation is a result of different homeostatic set-points among individuals of the group. Effects of age, sex, breed, reproductive status, and geographic location can contribute. The more broadly a population is defined, the greater the expected variation. Consider the anticipated inter-individual variation in blood values among all dogs (larger CV_G) compared to the inter-individual variation in blood values among laboratory-bred beagles (smaller CV_G). Inter-individual variation is reflected by the range (high minus low) of population-based RIs.

C. Intra-individual and inter-individual variation are typically determined by repeated measurement of an analyte in defined populations, and because a sample and a measurement are involved, the measurement of biological variation is affected by preanalytical and analytical variations. Preanalytical variations arise from preparation of the individual, sample collection, and the conditions from collection to testing, all factors that can be controlled by adherence to defined processes. Analytical variation for a particular assay is the imprecision of that assay's measurement (CV_A).

D. *Index of individuality (II or IoI, lol!)*: If assay imprecision (CV_A) is relatively small compared to CV_I and CV_G, which is the case for high-quality, well-controlled analyses, then the II of an analyte is the ratio of its intra-individual biological variation to its inter-individual biological variability: CV_I/CV_G.
   1. A high II (e.g., > 1.4) means that there is more intra-individual variation than inter-individual variation, so there is low individuality (individuals do not differ a lot from the group).
   2. A low II (high inverse II) means there is less intra-individual variation than inter-individual variation, so each individual's value fluctuates relatively mildly around a set point that varies more among individuals. A low II therefore reflects greater individuality.
   3. Note: The *inverse index of individuality*, which is determined by switching the numerator and denominator in the II calculation, is more intuitive, because a greater inverse II reflects greater individuality ($CV_I < CV_G$), and vice versa.

E. Procedures for determining CV_I, CV_G, II, and homeostatic set points are available,[3,5] and estimates of biological variation of various analytes in various species have been reported (www.vetbiologicalvariation.org). However, estimates of CV_I, CV_G, CV_A, and II vary considerably, thus limiting clinical application.

V. Impact of variation on data interpretation

A. Detecting disease by laboratory tests is based on recognizing when test results exceed expected degrees of variation, i.e., values are higher (increased) or lower (decreased) than expected. Recognition of unexpected findings is based on RIs that describe the combined biological variation in the reference population and the analytical variation of the assay. Unexpected values may reflect disease, an unusual biological variation, or an error (see Quality of Laboratory Results, I. Sources of Error).

B. Population-based RIs (see Reference Intervals, next section), which reflect the variation in analyte results across a group of subjects within a reference population, are commonly used to identify unusual results in individuals. These RIs work well for analytes with low individuality (individuals do not differ a lot from the group). However, population-based RIs are problematic for analytes with a high degree of individuality, because clinically important changes for an individual can be inapparent when values are compared to the broad population-based RIs that reflect the relatively large variation of the group (CV_G). For analytes with high individuality (e.g., creatinine), it is better to compare results to the individual's prior results, ideally by establishing a homeostatic set point and reference change value.[5,6] The number of samples needed to do so depends upon an analyte's CV_I and CV_A.

C. Reference change value (RCV) (also known as critical difference):[3,5,7] A RCV is the magnitude of change between serial results that is great enough to be considered of potential clinical significance, i.e., the change is greater than expected for estimates of biological and analytical variation. The value assumes negligible preanalytical variation. Changes in an analyte that exceed a provided RCV may support that a patient's status is improving, stable, or declining. Use of RCVs is limited.

REFERENCE INTERVALS

I. Reference intervals and their purpose
   A. Results of laboratory tests (laboratory data) on patient samples would be very difficult to interpret without *reference intervals*, which are usually population-based and reflective of the results we expect to find in most healthy animals of that population. These intervals are primarily used to help detect pathologic states. Other terms that are used as less-desirable synonyms include *normals*, *normal values*, *normal range*, and *reference range*.
   B. The following terms and definitions have been approved by the International Federation of Clinical Chemistry, the World Health Organization, and other groups.[8]
      1. *Reference individual*: an animal selected by using defined criteria
      2. *Reference population*: the group of all possible reference individuals
         a. Usually, the number of such individuals is unknown.
         b. In the case of captive wild animals, the total number of animals may be known.
      3. *Reference sample group*: an adequate number of reference individuals selected to represent the reference population
      4. *Reference value*: a value (result) obtained by observation or measurement of a particular substance in a reference individual
      5. *Reference distribution*: the distribution of reference values, which is sometimes Gaussian (a bell-shaped curve) and sometimes skewed
      6. *Reference limits*: the lowest value (LRL) and the highest value (URL) of the RI, as derived from a reference distribution
      7. *Reference interval*: an interval between and including the two reference limits (when only an upper limit is provided, e.g., < 3.5 µg/L, the lower limit of the RI is 0 or the detection limit of the assay)
      8. *Observed value*: a value obtained by observation or measurement that is to be compared to the RI
   C. Use of the term *reference range* is common but discouraged for two reasons.
      1. Statistically, a *range* is the difference between highest and lowest observations rather than the interval itself, e.g., the range is 40 if the highest observation was 50 and the lowest was 10.
      2. It may be ambiguous whether "reference range" refers to the RI or includes low-to-high values for all the reference individuals, about 5 % of which are excluded from typical RIs.
   D. Using the terms *normal* and *abnormal* to describe laboratory test results is common but can be misleading and is discouraged.
      1. A laboratory result can be WRI but still reflect a pathologic process, so it would not reflect normality in that individual. For example, a serum [Na^+] that is WRI in a dehydrated animal indicates that the animal has lost both water and sodium from its body.
      2. Healthy animals are expected to have some laboratory results outside of the RI because typical RIs exclude approximately the upper and lower 2.5 % of reference values. Such excluded values are outside the RI but do not reflect an abnormality.
      3. Because RIs are determined from a subset of the reference population, reference limits are merely a statistical estimate of the limits of health, but they do not define health or normality.
      4. It is difficult to define "normal" because many variations that may appear to be "abnormal" are caused by physiologic, dietary, environmental, or other nonpathologic factors.

II. Establishment of reference intervals: Establishing de novo (from scratch) RIs is a major laboratory undertaking that should not be approached without consideration of the time, expertise, and financial implications for the laboratory. The testing alone can add up to many thousands of dollars, and hundreds of hours of professional time are dedicated to establishing RIs for four major veterinary species before even considering the variety of other species (e.g., camelids) and subgroups (e.g., juveniles and geriatrics). The following major steps in the process help limit variation in RI results to interindividual variation within a given reference population that is assessed by given methods. Readers are referred to other publications for further details.[9,10]

A. Select and record inclusion and exclusion criteria for reference individuals. Criteria include species, age, sex, and method of determining health status.

1. Inclusion criteria should reflect the demographics and diversity of the population for which the RIs will be used.
2. Exclusion criteria should ensure that unrepresentative and ill subjects are excluded from the reference sample group so as not to contaminate the RI data. Neonates and young animals are typically excluded from routine RIs because of age-related differences in some analytes; separate RIs may be generated for these groups. Certain breeds with atypical results for certain analytes are excluded for the affected analytes (e.g., [platelet] in greyhounds).
3. In domestic species, determination of health may be as limited as a history and physical exam or as thorough (and expensive) as a full medical evaluation with screening blood work and imaging. How would you assess and document health in free-ranging wildlife?
4. Establishment of inclusion and exclusion criteria should include consideration of potentially clinically relevant differences that occur because of variations in breed, physiologic state (including pregnancy and lactation), geographic location (including altitude), nutritional state (including diet), and subclinical disease (e.g., retroviral infections). Should a reference sample group for dairy cattle include milking cows, dry cows, steers, and bulls?

B. Establish an appropriate size of the reference sample group, preferably to have at least 60 animals that meet selection criteria.[11] This typically requires sampling of several more individuals than the goal because some will be excluded when found to have subclinical disease or because of sample problems or other factors.

1. Appropriate sample size depends on reference population homogeneity and the distribution of the reference data. A smaller sample can be representative of a species that is fairly homogeneous (red fox), but a larger sample is required for a species that is heterogeneous (domestic dogs).
2. Some RI guidelines[10] call for at least 120 reference individuals, 120 being needed to calculate 90 % confidence intervals around nonparametric reference limits. However, reference sample groups of that size are often not obtained in veterinary medicine, and a more realistic number of 60 representative individuals may be sufficient with appropriate statistical analysis.
3. Obtaining representative samples from the desired number of qualified reference individuals is often a challenge. Consider the strengths and weaknesses of the following potential reference sample groups:
   a. Animals presented for yearly vaccinations, elective surgical procedures, or as part of a blood donor program; these groups may overrepresent young animals or large-breed dogs
   b. Collecting many samples from one kennel, one cattery, one stable, or one herd; the similar genetics, environment, and nutrition of such groups may yield results that are not representative of the population of interest

C. Collect and process the samples. The sample collection and processing protocol should be defined and aligned with what is used for clinical samples. Samples that do not meet collection and processing requirements should be excluded. Consideration should be given to the following:
   1. The need for fasted versus nonfasted samples, and the duration of fasting
   2. Method(s) and site(s) of collection (e.g., jugular versus cephalic vein versus indwelling catheter, vacuum tube versus syringe); should collection be uniform or varied to the degree that occurs with samples to be evaluated?
   3. Type of sample tube/container or anticoagulant and volume of the sample
   4. Storage time and temperature of samples prior to testing
   5. Sample quality issues such as substantial hemolysis, lipemia, or any clots in an anticoagulated sample

D. Measure or determine the reference values. Analyze the sample for the substance(s) of interest (analyte) in the same manner as patient samples, including use of appropriate QC measures (see Quality of Laboratory Results, III.B).
   1. Concurrent assessment of routine blood test panels (e.g., CBC, chemistry, blood gas) has financial and efficiency benefits, including health screening and determination of multiple RIs from a single reference sample group.
   2. Test results are inspected to identify reference subjects that may have subclinical disease or sample results with preanalytical or analytical error. This is ideally done as results are generated so that correctible errors can be addressed, and further testing can be done to evaluate for disease. Whether done real-time or later, data from individuals with evidence of disease are excluded in toto or in part, the latter if abnormalities have a known cause that does not affect other values.

E. Determine the reference distribution. Begin by displaying the data in a histogram and assessing by visual inspection. Apply statistical methods, such as the Shapiro–Wilk goodness-of-fit (normality) test, to determine whether data have a Gaussian (normal) distribution.[12]
   1. Many analyte results fit a Gaussian distribution, especially those that are tightly regulated by physiologic systems (e.g., concentrations of glucose, Na^+, K^+, and fCa^{2+}).
   2. Many analyte results (e.g., serum enzyme activities) do not fit a Gaussian distribution; these typically have positive (tail to the right) or negative (tail to the left) skewness.

F. Identify and remove outlier values, as these inappropriately affect the reference limits.
   1. Outliers are data that do not fit into the rest of the distribution. They may result from inadvertent inclusion of clinically or subclinically ill subjects or from preanalytical, analytical, or postanalytical errors (see Quality of Laboratory Results, I. Sources of Error).
   2. Statistical outlier tests are available to assess single or multiple (one at a time or sequentially) outlying values at one or both ends of the reference distribution.[13] Some are parametric tests requiring mathematical transformation of nonGaussian to Gaussian data.
   3. The cause of outliers should be investigated. They might result in exclusion of the single result or the entire reference subject because of illness, or they might be corrected after identifying an error. There should be a clear and convincing rationale for concluding that a value is not representative of health and therefore should be excluded; values should not be excluded simply to render the distribution Gaussian, as this will inappropriately affect reference limits.
   4. After outliers are excluded, the distribution of the data should again be determined.

G. Determine reference limits and RIs. The most commonly used RIs represent exactly or approximately the central 95 % of reference values.[14]

1. Limits may be defined by excluding the top and bottom 2.5 % of values. If 2.5 % of the values are > 150, and 2.5 % of values are < 50, the reference limits are 50 (LRL) and 150 (URL) and the RI is 50–150. This nonparametric approach has been recommended whenever the sample size is at least 120. It can be used with as few as 40 samples. With < 40 reference samples, reference limits are represented by the highest and lowest reference values. However, 90 % confidence limits of the reference limits cannot be calculated nonparametrically with fewer than 120 samples. When fewer than 120 reference samples are available, 90 % confidence limits for nonparametric RI must be calculated using bootstrap (resampling) methods.
2. When data have been shown to fit a Gaussian distribution, parametric methods may be used to establish reference limits based on the mean ± 2 SD of values (the central 95.54 % of values rounded to appropriate significant digits). Confidence intervals of the reference limits can be determined by parametric and bootstrap (resampling) methods.
3. When the distribution is not Gaussian and the sample number is less than 120, the data may be transformed into a Gaussian distribution, e.g., using the Box–Cox method for right-skewed data,[15] and assessed parametrically.
4. An iterative or resampling method called the robust method can be used to determine reference limits for large and small data sets. This method performs best if the data are symmetrically distributed, but Gaussianity is not required.[16]
5. Figure 1.2 shows the differences between Gaussian and skewed distributions and the RIs obtained from such distributions. Box–Cox transformation of the skewed distribution might convert it to Gaussianity.

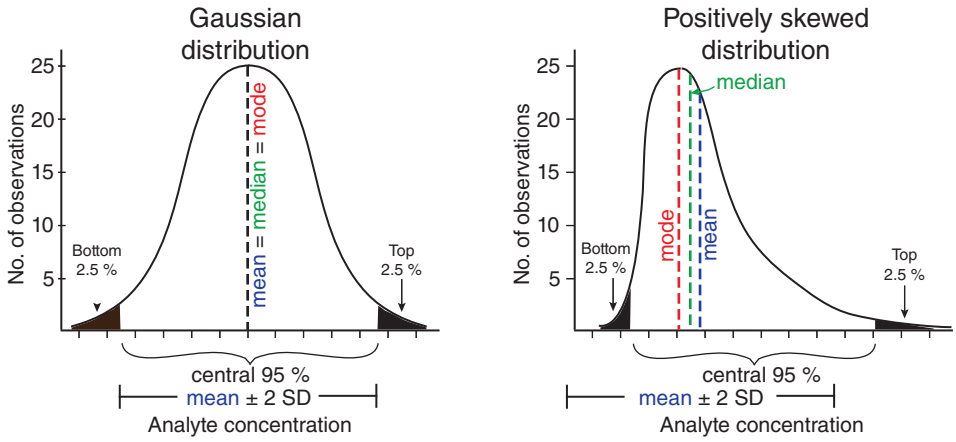


Figure 1.2. Reference distribution.

- In the left graph, the reference values conformed to a Gaussian distribution. If data have this distribution, then mean ± 2 SD represents the central 95 % of the reference values and thus the RI. If values have a Gaussian distribution, the mean, median, and mode values are equal.
- In the right graph, the reference values had positive skewness. If data have this distribution, nonparametric methods are used to determine the central 95 % of the values. If the interval represented by mean ± 2 SD were calculated, the LRL would be below the lowest reference value, and the URL would exclude more than the top 2.5 % of the reference values. Therefore, mean ± 2 SD would not be an accurate representation of the values expected in healthy animals. The mean, median, and mode values will be different if the reference values have a nonGaussian distribution. Statistical transformation of these data might yield a Gaussian curve that could be used to calculate reference limits.

H. Calculate 90 % confidence intervals (CIs) of the upper and lower limits.[17] Confidence intervals around the limits describe the precision of the limit estimates.
I. Document details of RI studies in a comprehensive report that can be made available to users upon request.
J. RIs should be verified every 3–5 yr.[9] Several procedures for verification are available, including the simple method described in the following section regarding transference. Methods using archived laboratory results also are described.[18]

III. Transference (adoption) of existing RIs
A. Transference of existing RIs from one laboratory to another laboratory or from one analyzer to another analyzer is a quicker and less expensive alternative to establishing de novo RIs. This may be done when analyzers are updated to new models or new methods are added, but it should be limited to a single occurrence, especially if the RIs require adjustment for bias between methods or analyzers. Once the method or analyzer is established in the laboratory, results from healthy subjects can be collected over time for de novo RIs.
B. Sources of RIs for transference may include the laboratory itself, another reputable laboratory, or the manufacturer of the analyzer or method.
C. Adherence to the following steps helps ensure that transferred RIs are appropriate.
1. Confirm that the transferred RIs match the adopting laboratory's patient population and collection methods. This requires availability and review of the donating laboratory's comprehensive RI study document.
2. If RIs are not transferred between the same methods and type of analyzer, perform a comparison of methods study (see Evaluating and Validating Laboratory Methods, Section IV) and adjust reference limits if there are biases that exceed predetermined quality goals (e.g., TE_a).
3. Confirm similar precision and accuracy of the analyzers and methods.
4. Verify the transferred RIs using samples from the laboratory's patient population. This can also be done to verify factory RIs provided for an analyzer. The simplest procedure is as follows:
a. Collect and analyze samples from 20 healthy subjects using the same inclusion and exclusion criteria established for de novo RIs.
b. Generally, accept the RI if two or fewer results fall outside it – theoretically, 95 % (19 of 20) would be WRI – but if no results fall outside the RI and they are clustered in a limited region, the RI might be too broad.
c. Reject the RI if five or more results fall outside it.
d. If three or four results fall outside the proposed RI, collect and assess an additional 20 samples from healthy subjects. Accept the RI if two or fewer results fall outside it; otherwise, reject it.

IV. Use of RIs
A. It is important to understand that most RIs represent results expected in approximately 95 % of healthy animals (i.e., in 19 of 20 healthy animals).
1. Therefore, 1 of 20 healthy animals is expected to have a measured value outside of the RI. The value that is outside the RI but still represents health is expected to be close to a reference limit. A marked difference from the RI probably represents a pathologic state, although it could also represent an erroneous value.
2. When examining multiple test results for one animal, it is important to remember that this 95 % chance of a value from a healthy animal falling WRI applies to each result

separately. (Note: The chance is theoretically 95.54 % for parametric methods, but not exactly given rounding to significant digits.)

a. As shown below, this means that in a panel of 20 assays, each with a 5 % chance of being outside of the RI even without pathologic change, there is a 64 % chance for at least one value to be outside the RI. With a panel of five results, there is about a 23 % chance. However, such values should be close to the lower or upper reference limit. This concept is particularly important when laboratory tests are requested for clinically healthy animals for a yearly health profile or a geriatric profile. Also, it would be unexpected for all CBC and chemistry results in a study's healthy control group to be WRI, if RIs were appropriate.

b. These percentage chances are derived from probability calculations. Because there is about a 95 % (0.95) probability in healthy animals that each result is WRI, the probability that all n values are WRI is 0.95^n, and the probability that not all values are WRI (i.e., at least one is not WRI) is $1 - 0.95^n$ (where n is the number of observations). Thus, for a panel of 20 test results, $100 \times (1 - 0.95^{20}) = 64$ %, so there is a 64 % chance that at least one value is outside the RI.

B. Not all RIs accurately reflect expected findings in a population of interest. This may be because of such problems as an inadequate procedure of establishing the values, a difference between reference and test populations, or a change in methods or instrumentation by the laboratory without an appropriate change in RIs.

C. It is appropriate to ask a laboratory for the basis of their RIs. This enables evaluation of how likely it is that the values reflect health in the population of interest. Such requests are particularly appropriate when first contemplating use of a laboratory. Requested information might include the following:

1. Number of reference individuals
2. Ages, breeds, and genders of reference individuals
3. Criteria for inclusion as a reference individual
4. Methods for establishing RIs, including de novo versus transference

D. Use of RIs published in textbooks is discouraged but may be necessary to provide guidelines for certain populations (e.g., neonates, wildlife, zoo animals). Such RIs may not be applicable because of preanalytical and analytical variations, or because the reference population does not represent the individuals of interest. Unfortunately, for many textbook RIs, important details may not be documented.

V. Reference limits versus decision thresholds (limits)

A. As defined above (see Reference Intervals, Section I.B.6), reference limits are the highest value and lowest value of the RI and thus are descriptive of a reference distribution; a RI includes values expected in about 95 % of the healthy animals. These values are used diagnostically to help detect pathologic states in ill animals. A patient value that is above or below the reference limits might indicate the presence of a pathologic state.

B. A *decision threshold* (clinical decision limit, cutoff point, or cutoff value) is a value that is used to classify a result as positive or negative for a disease, or to decide whether to treat or not to treat.[19] In other words, decision limits help *discriminate* between subjects with or without a particular disorder. As described in the section on Diagnostic Properties and Predictive Value of Laboratory Assays, a decision threshold is not necessarily a reference limit. Decision thresholds can also be used to estimate a cost-to-benefit ratio for diagnostic methods. Choosing a higher or lower decision limit alters rates of false positive or false negative conclusions, thus affecting costs related to additional diagnostics, inappropriate patient management, and increased morbidity or mortality.

C. The term *decision limit* is also used in the context of a *critical decision limit* or critical value, which is a value that is used for making critical therapeutic decisions. For example, a critical decision limit for serum [tCa^{2+}] might be 15.0 mg/dL, meaning that aggressive therapy is initiated whenever a serum [tCa^{2+}] is greater than 15.0 mg/dL. In contrast, the upper reference limit might be 12.0 mg/dL, and a decision threshold for a particular hypercalcemic disorder might be 12.5 mg/dL.

QUALITY OF LABORATORY RESULTS

I. Sources of error
   A. When results are unexpected, a clinician might say, "I don't believe these results; there must have been a lab error." It is important to remember that there are many potential reasons for an erroneous laboratory test result, and identifying the source requires recognition of where errors can occur throughout the laboratory cycle (Figure 1.3). Errors anywhere along the cycle may jeopardize patient management, and many do not occur in the lab. Errors may be preanalytical, analytical, or postanalytical.
   B. *Preanalytical errors* are common (Table 1.7) and arise from problems prior to sample analysis. They may occur before or after the sample reaches the laboratory, and include errors in patient preparation, test selection, sample collection, and sample processing.[20] These errors can be minimized with laboratory guidance and attention to the following:
      1. Patient preparation: Properly prepare the patient, including fasting many small animal patients and minimizing excitement prior to sampling.

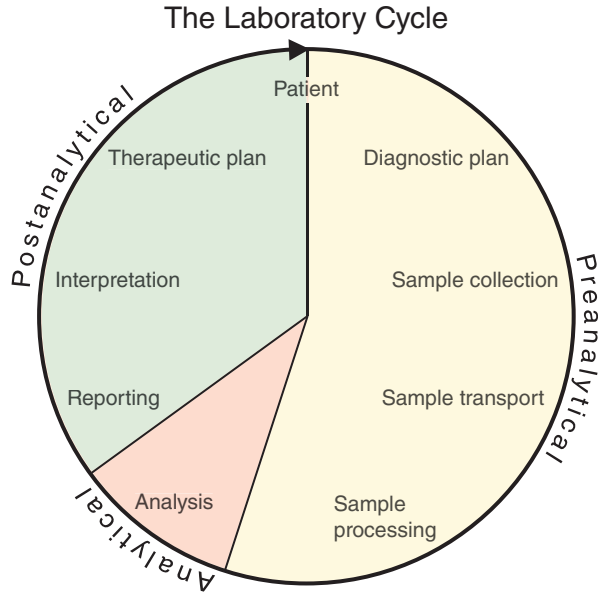


Figure 1.3. The laboratory cycle. Laboratory testing begins with the patient (*top*). After establishing a diagnostic plan, samples are collected, transported, and processed for analysis. Generated results are reported, and their interpretation leads to a therapeutic plan that is applied to the patient. Errors can occur at any step in this cycle, preanalytical errors occurring prior to analysis (*yellow region*), during analysis (*pink region*), or after analysis (*green region*). When using accredited laboratories, the fewest errors occur during analysis.

Table 1.7. Examples of preanalytical laboratory errors[a]

Prelaboratory	Prelaboratory (continued)
Test selection	Sample transport
Intentionally or accidently choosing wrong test	Delayed transport to laboratory
Sample collection	Sample held at improper temperature
Sample collected after treatment instituted	Sample not protected from breakage
Traumatic venipuncture	Regulatory noncompliance for biologicals
Prolonged venipuncture into syringe	Unstained smears exposed to formalin
Catheter or port improperly flushed	Submitted information
Blood tube underfilled	Unnecessary test requested
Urine collected from contaminated surface	Request not clear
Cytologic sample not representative	Species not provided
Collected too superficially	Fax, phone, address not provided
Fluid or necrotic center collected	For cytology: lesion not described
From single region of large mass	Reason for testing not indicated
Bone marrow aspirate hemodiluted	Multiple samples not clearly identified
Sample container	Bone marrow sample lacks CBC results
Urine collected into contaminated container	
Wrong blood tube used	
Tube past expiration date	
Sample processing[b]	
Blood tube not inverted promptly and properly	**In the laboratory**
Tubes and slides not labeled	Delayed processing (e.g., urinalysis)
Fresh blood smears not made (CBC)	Sample accepted that should be rejected
Blood smears thick or otherwise problematic	Sample mix-up or misidentification
Fluid sent out without fresh preparations	Centrifugation force/time inappropriate
No concentrated preps of low-cellularity fluids	Serum removed before thorough clotting
Cytologic sample sprayed on slide	Plasma and serum mixed
Cytologic sample not spread on slide	Cell contamination when serum harvested
Bone marrow smears thick or lack particles	Processed at inappropriate temperature

[a] Many examples apply whether testing is in-clinic or send-out; some apply only to send-out. If testing is in-clinic, clinic personnel must avoid errors; when sending samples out, trained laboratory personnel will help prevent or recognize problems.

[b] See "In the laboratory" for examples of other processing errors that may also occur prior to sending samples out.

2. Sample collection: Use proper collection order and technique to collect appropriate sample types and amounts for testing and potential retesting or additional testing (seek lab guidance as needed).
   a. Sample directly to vacuum tubes when possible; collection into a syringe with subsequent and delayed filling of tubes promotes clotting, platelet activation, and problematic samples.
   b. To obtain 1 mL of serum, at least 3 mL of blood is usually needed.
   c. Fill citrate tubes enough to yield a 9:1 blood to anticoagulant ratio.

3. Sample container: Use appropriate container and contents.
4. Sample processing: Mix samples as recommended, label samples, follow appropriate processing guidelines, make good smears of fresh samples, and avoid or minimize testing delays.
5. Sample transport: Transport promptly at appropriate temperature, maintain separation of cells and serum, package to avoid breakage, and keep formalin fumes separated from unstained smears.
6. Sample submission: Request appropriate test, provide all relevant client information, clearly identify all samples, and provide appropriate patient information.
7. Appropriate laboratory processes: Process samples appropriately, reject unsuitable samples, and confirm accurate sample identification throughout.

C. *Analytical errors* occur during analysis of the sample (Table 1.8) and should be minimized by attention to the following:
1. Using and maintaining quality instruments and equipment: Generally, "you get what you pay for," but a quality instrument remains a quality instrument only if it is properly maintained.
2. Minimizing operator errors: Have personnel properly trained and use written standard operating procedures to help avoid person-to-person variation.
3. Using instruments and methods appropriate for the species: An instrument calibrated to measure the relatively large human RBCs may not provide accurate measurement of smaller RBCs from domestic mammals.
4. Using appropriate reagents and consumables: Store and use according to instructions, including disposal of expired reagents.
5. Using a quality assurance plan (QA program): Laboratory personnel should adhere to internal assessment procedures to minimize, detect, and resolve errors to help ensure that all parts of the quality analysis of the sample are maintained daily.[21]
6. Recognition and, when possible, avoidance of interferents of laboratory results for the methods being used: Interferents include various therapeutics or their metabolites, hemoglobin from hemolysis, lipemia, and bilirubin.[22]
    a. Drugs may alter laboratory results because of their physiologic or pharmacologic effects, but some drugs or their metabolites cause direct analytical interference

Table 1.8. Examples of analytical errors

Instrument or equipment malfunction	Reagent problem
Physical problem	Reagents expired
Electronic problem	Bad lot of reagents
Out of calibration	Reagents improperly stored/handled
Operator error (consistent or sporadic)	Results accepted without acceptable control results
Untrained operator mistake	Interferences
Tired or distracted operator mistake	Lipemia
Operator short-cuts/alters procedure	Hemolysis
No standard procedure to follow	Hyperbilirubinemia
Wrong/old SOP[a] followed	Certain treatments
Wrong procedure	
SOP contains error(s)	

[a] SOP, standard operating procedure

(e.g., potassium bromide treatment falsely increases [Cl^-] and falsely decreases [glucose] by certain methods). Collect samples pretreatment when possible.

b. Hemoglobin may interfere with certain wavelengths of light transmission in spectrophotometric assays and yield false increases or decreases, depending on assay design. Avoid causing in vitro hemolysis.

c. Bilirubin in icteric serum or plasma may interfere with certain wavelengths of light transmission and alter results of some spectrophotometric assays.

d. Lipoproteins in serum, plasma, or other fluids can cause turbidity and therefore interfere with light transmission used for many assays. They can also alter protein conformation, which may affect some immunoassay and electrophoresis results, and they increase the volume percentage of solids in samples, thereby falsely decreasing electrolyte concentrations measured by indirect potentiometry.[23] Postprandial lipemia can be avoided by appropriate fasting.

D. *Postanalytical errors* occur after analysis, either within the laboratory or outside it (Table 1.9). They can be minimized with attention to the following:

1. Accurate entering and transferring of data: Limit need for data transcription and ensure care during transcription of results to a laboratory report or during keyboard entering of data into computers.
2. Verifying results and reports: Inspect reports before release.
3. Prompt, clear, and complete reporting to the correct destination: Minimize hand-written reports.
4. Appropriate use of RIs: Recognize that provided RIs may not be appropriate for a particular sample, e.g., one from a neonate.
5. Appropriate interpretation of results: Do not assume unexpected results are laboratory errors; use resources and continuing education opportunities.

II. Analytical properties of assays

A. Analyzers and their maintenance impact the quality of laboratory results. From the analytical perspective, the best clinical laboratory assay is one that consistently measures the true concentration or activity of the substance and at concentrations and activities that are clinically relevant. When evaluating and comparing laboratory assays, five analytical properties can be assessed.[24]

Table 1.9. Examples of postanalytical laboratory errors

In the laboratory	After the laboratory
Transcription incorrect	Results or recommendations are ignored
Result validation/verification	Results are misread or misunderstood
No system for validation of results	Flagged analyzer results are not ignored
Error missed while validating	Results are present but not noticed
Reporting	Results are misinterpreted
Reported to wrong person/place	Laboratory not consulted for clarification
Report delayed	
Report illegible	
Report incomplete	
Reference interval error	
No reference values provided	
Poor reference intervals provided	

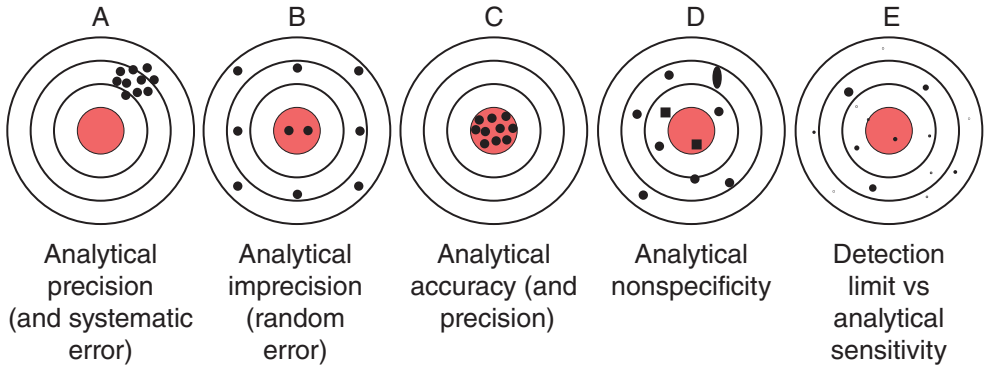


Figure 1.4. Illustrations of analytical properties of assays.

A. *Analytical precision (systematic error)*: Because the 10 holes in the target are tightly clustered, the target shooter was precise even though consistently (systematically) inaccurate.

B. *Analytical imprecision (random error)*: Because the holes in the target are evenly distributed, the average of 10 shots is exactly in the middle of the target area. Thus, the shooter was statistically accurate but imprecise.

C. *Analytical accuracy (and precision)*: Because all 10 holes in the target are tightly clustered in the middle of the target, the shooter was accurate and precise.

D. *Analytical nonspecificity*: Of the 10 holes in the target, 7 are round, 2 are square, and 1 is oval. Of the 10 observations, the shooter probably created 7 and 3 were created by other factors. Thus, the presence of holes in the target is not specific for the shooter's actions.

E. *Detection limit vs analytical sensitivity*: How many holes can you see in the target? The smallest hole that you can reliably detect is the detection limit of your eyes. Analytical sensitivity in this context is the smallest change in hole sizes that your eyes can reliably differentiate. If your eyes can reliably detect the different sizes of all 15 holes, then the sensitivity limit has not been reached. If your eyes can differentiate the changes in the four largest holes but not the other holes, the sensitivity limit is between the fourth and fifth holes. Each shot was made with a projectile whose diameter was 75 % of the previous shot.

B. *Analytical precision*: ability of an assay to get the same result if a sample is analyzed several times; this is also called repeatability, reproducibility, or random analytical error (Figure 1.4A)
   1. The need for analytical precision depends on the degree of variation that can be accepted as random variation (error). Typically, manual assays have more random error than do automated methods because people generally cannot reproduce their work as well as an instrument can. When management of a case requires that small changes in concentration reflect a biological change and not analytical variation, an assay needs to have high precision. However, if a wide range of measured values can be accepted, e.g., creatine kinase activity, then high precision is not needed.
   2. Precision is frequently expressed in clinical laboratories by the method's CV (Eq. 1.1). A method's CV is determined from replicate analysis of a sample or QCM within an assay run and between different runs of the same assay.[25]
      a. A *within-assay (within-run, intra-assay) CV* (also called repeatability) represents the random error that is expected when one sample is analyzed multiple times in one run of an assay. If an assay has a poor within-assay CV, the sample's analyte concentration should be determined by analyzing the sample in duplicate or triplicate and then calculating a mean concentration. However, repeated measures are not economical for routine testing in clinical pathology laboratories, so routine assays

generally have sufficient precision to provide reliable results when testing only once and monitoring with an effective QA program.

b. A *between-assay (inter-assay) CV* (also called reproducibility) represents the random error over multiple runs of the assay when using the same sample, which must be stable for the duration of the assessment. The within-assay CV is expected to be smaller than the between-assay CV; the opposite pattern suggests a problem with the precision studies.

c. The clinical relevance of an assay's CV is determined by many factors.

(1) If critical clinical decisions are made when changes in an analyte's concentration are minimal, then the assay must have a very low CV (high precision). Otherwise, the diagnostician would not know if the change in analyte concentration is due to a true change that occurred in the animal or if the change represents analytical error.

(2) An assay's CV typically varies with the analyte's concentration. Higher CV values may be found at the lower and upper limits of the assay's analytical range. Within the analytical range, CV values are typically higher at lower analyte concentrations because CV values are expressed as percentages. This is most apparent for analyte quantities that vary the most.

(a) If an analyzer's [platelet] measurement has a SD of 3000/µL at all concentrations, the assay's CV values would be 50 % at a mean concentration of 6000/µL, 5 % at a mean of 60,000/µL, and 0.5 % at a mean of 600,000/µL.

(b) If a [platelet] assay has a CV of 5 % at all concentrations, then the assay's SD would be 300/µL at an analyte concentration of 6000/µL, 3000/µL at an analyte concentration of 60,000/µL, and 30,000/µL at an analyte concentration of 600,000/µL. Depending on the analyte and the amount of its variation within an individual (biological variation), a CV of 10 % can be unacceptable or acceptable analytical variation (see TE_a in III.B.5 and III.B.8 of Quality of Laboratory Results).

(3) Knowing the amount of analytical variation for an assay is helpful when trying to determine whether a change in a patient's data represents analytical variation or a true biological change. One way to approach this decision is to use an assay's USD. *USD* is an average of SD values from three to six consecutive months of QA values. If a change in a patient's data is less than twice the USD, the change may be only analytical variation. If a change in patient data is greater than three times an assay's USD, then the change is probably truly reflecting a biological rather than analytical cause. The cumulative average SD representing USD varies considerably across assay methods but is often accessible to laboratorians through analyzers' onboard monitoring systems.

(a) If the USD for $[Na^+]$ at 150 mmol/L is 1.5 mmol/L and a patient's $[Na^+]$ is 150 mmol/L on day 1 and 148 mmol/L on day 2, then the change of 2 mmol/L may be due to analytical variation since it is less than twice the USD. However, a value of 145 mmol/L on day 2 (a change greater than three times the USD) probably represents a true biological change.

(b) If the USD for blood [neutrophil] is 1000/µL at a [neutrophil] of 10,000/µL, then the [neutrophil] in a second sample needs to be < 7000/µL or > 13,000/µL to be reliably considered a biological change if the first sample had a [neutrophil] of 10,000/µL.

(4) If a change in serial values appears to be biological, that does not mean it is of clinical significance because it might be within expected limits of biological variation. As discussed in the Analytical and Biological Variation section, V.C, RCVs or critical differences might become useful for assessing clinical significance when appropriate data are available. Other approaches to determining and conveying measurement uncertainty for laboratory data include measurement uncertainty (MU), expanded measurement uncertainty (EMU), and dispersion.[26–29]

d. An assay's precision (CV_A) has an impact on how results are reported, including the number of significant digits (see Significant Digits) used and the reporting interval. *Reporting interval* is the increment used to report laboratory results for an analyte, most often in decimal units of 0.01, 0.1, 1, or 10, although nondecile increments can be used.[29] Use of appropriate significant digits and reporting units helps communicate the uncertainty of a result. Rules for selecting clinically appropriate reporting intervals based on analytical variation and critical differences have been published.[26,30]

C. *Analytical accuracy*: closeness of the agreement between the measured value of an analyte and its "true" value (Figure 1.4C)

1. Methods of establishing "true" values vary considerably. At times, the true value is established by a reference method (e.g., a method developed by the NIST). If such standards have not been developed, the "true" value might represent the mean concentration determined by numerous observations using an assay that is accepted as the best available.
2. Typically, accuracy of a clinical assay is assessed by comparison of its results to the results of an accepted reference method, a method that has been accepted by a standardization group as providing a true value. Optimally, the assay that is accepted as the reference method is also precise so that only a few observations are needed to determine the true value (Figure 1.4C).
3. If method comparison reveals biases and results that deviate from those of a good reference method, the assay is not accurate. Use of a more accurate method or analyzer would be indicated, if available. Establishing reasons for the inaccuracies may allow assay modification, but these may need to be done by a company as part of analyzer development. Although adjustment of RIs from inaccurate assays is commonly done to allow interpretation of inaccurate data, this practice contributes to interlaboratory variations and impairs use of broadly acceptable RIs and medical decision limits.
4. Standard solutions
   a. The accuracy of the clinical assay may be assessed by measuring an analyte's concentration in a *reference standard solution* whose concentration was certified after determination by a reference method.
   b. Reference standard solutions are not the same as calibration standard solutions. *Calibration standard solutions* are commercially prepared and used to calibrate an instrument or method for a particular analyte. They should closely agree with reference standard solutions.
   c. In contrast to calibration standards, *quality control materials* (QCM) are analyzed every day or shift to monitor analytical performance by comparing measured values to expected results. They contain one or more analytes at concentrations that may be below, above, or within RIs. QCM may be "assayed" or "unassayed;" "assayed" QCMs have assigned analyte concentration intervals determined by the manufacturer, usually on several analyzers. For "unassayed" QCM, analyte concentrations are established through repeated measurement by the user.

D. *Analytical specificity*: ability of an assay to detect only the substance of interest (analyte); freedom from interfering substances (Figure 1.4D)
   1. Analytical specificity is related to analytical accuracy because an assay cannot be accurate if a nonspecific reaction is occurring.
   2. The need for analytical specificity varies directly with the likelihood of interfering substances. A serum glucose assay may be designed to react with all hexoses. Such specificity may be acceptable if glucose is the only hexose in serum that is at a sufficient concentration to be detected by the assay. Other glucose assays may be designed to react only with glucose even if other hexoses might be present in the sample.
   3. Substances might interfere with an assay in many ways:
      a. The substance might be very similar chemically, so the assay reacts with either the analyte or the interfering substance (e.g., Br^- detection falsely increases $[Cl^-]$).
      b. The substance might produce the same response that is detected in the assay system.
         (1) The presence of glucose might be detected when a chemical reaction produces hydrogen peroxide (H_2O_2). A substance can interfere with the assay by having the same oxidizing properties as H_2O_2.
         (2) In spectrophotometric assays, the presence of lipids, bilirubin, or hemoglobin can interfere with light transmission through a sample, and thus the results of the assay are changed by artifactual spectral changes and not by a chemical reaction.
   4. Depending on how a substance interferes with an assay, it might lead either to falsely increased or to falsely decreased concentrations, i.e., positive interference or negative interference, respectively. The degree and direction of interference can vary with the method, even for a single analyte.

E. *Detection limit* (definition of the International Union of Pure and Applied Chemistry, IUPAC): the smallest concentration or quantity of an analyte that can be detected with reasonable certainty for a given analytical range (Figure 1.4E)
   1. An assay's detection limit involves the ability of the assay to differentiate background "noise" from a true change because of the presence of an analyte. The detection limit defines the lowest value of an assay's analytical range; *analytical range* is the range of values over which the assay can provide reliable results.
   2. If an analyte is relatively abundant (e.g., serum Na^+), then a detection limit of 100 mmol/L may be adequate. For substances that are relatively rare (e.g., aldosterone), a detection limit of 100 pmol/L may be needed to be clinically useful. One pmol is a billionth of 1 mmol.
   3. Note: Several different terms and definitions are used for this general property.

F. *Analytical sensitivity* (definition of the International Union of Pure and Applied Chemistry, IUPAC): the slope of the calibration curve and the degree to which an analytical procedure produces a change in the signal for a defined change of the quantity (however, some organizations and many people consider analytical sensitivity to reflect detection limit)
   1. In other terms, an assay's analytical sensitivity is how much change of the analyte (concentration, property, etc.) is needed for the assay to detect the change. For example, an assay that can differentiate 50 mg/dL from 51 mg/dL has better analytical sensitivity than an assay that can only differentiate 5.0 g/dL (5000 mg/dL) from 5.1 g/dL (5100 mg/dL).
   2. Analytical sensitivity should not be confused with detection limit. Both relate to small changes in concentration, but, as defined by IUPAC, analytical sensitivity applies to changes within an assay's analytical range, whereas detection limit applies to the lowest limit of the analytical range.

III. Quality assurance

A. Every clinical laboratory (from large reference laboratories to small in-house laboratories) should have a QA program to monitor laboratory performance and ensure that reliable results are generated.[31] The Quality Assurance and Laboratory Standards Committee of the American Society for Veterinary Clinical Pathology is dedicated to the education and communication of quality laboratory topics and has published guidelines for the monitoring of laboratory procedures (www.asvcp.org/page/QALS_Guidelines).[32–40] Quality assurance programs encompass all nonstatistical and statistical procedures implemented to minimize error in laboratory results. When suitable control materials are not available (e.g., for urinalysis sediment examination), one must rely on nonstatistical QA methods.

1. Nonstatistical QA components include:
   a. Written standard operating procedures (SOPs)
   b. Maintenance routines with log-sheet documentation for all equipment
   c. Personnel with appropriate training and continuing education
   d. A responsible laboratory supervisor
   e. Internal or external proficiency testing
2. Statistical QA processes are often referred to as *quality control* (QC) testing and encompass testing of QCMs to ascertain presence or absence of analytical errors caused by problems with (i) the operator, (ii) the reagents, (iii) other consumables, or (iv) the instrument. To detect relevant operator errors, QC testing should be done by the person testing patient samples. Detectable analytical errors can be random or systematic.
   a. *Random error* is imprecision caused by factors that randomly affect the measurements, such as variations in dispensed volume of reagent or sample (Figure 1.4B).
   b. *Systematic error* (*bias*) is reproducible inaccuracy that consistently results in values that are too high or too low; causes include deterioration of a reagent or a light source (Figure 1.4A).
   c. QC testing does not assess for analytical error caused by interferents in patient samples, and it does not assess for preanalytical or postanalytical errors. If a so-called electronic QC-check is done rather than assessment of an actual QCM sample, that can verify only that an instrument's circuitry is functioning; it does not assess the entire operation of the analyzer from sample application to reporting a result.

B. Quality control testing

1. Quality control testing should be designed to detect random and systematic analytical errors of clinical significance before patient results are determined or reported. To this end, QCMs (see Quality of Laboratory Results, II.C.4.c) are generally assessed for each test at the beginning of each day or shift prior to testing patient samples. QCMs may contain analytes at quantities that are below, within, or above RI to verify that the assay can measure values that are decreased, WRI, or increased; these QCMs can be tested on a rotating schedule with only one per analyte each day.
2. If single and serial results for a QCM are within defined limits, then the assay appears to be "in control" and performing correctly, thus patient results are probably valid and can be generated and reported. If QCM results are outside the defined limits, then the assay is "out of control!" and troubleshooting is indicated to identify a source of unacceptable analytical error, i.e., operator, reagents/consumables, or analyzer. Although care should be taken to manage QCMs so that they are reliable, it is also possible that the QCM is the problem, so assessment of a new QCM might be indicated if other errors are not

found. Reliable patient results cannot be generated until corrective actions resolve the error, as demonstrated by subsequent QCM results being within defined limits.

3. *Westgard rules*, developed by James O. Westgard, Ph.D., form a system of rules that are applied singly or in combinations to warn of unwanted random or systematic error in QCM results (www.westgard.com).[41] Example rules follow; "s" stands for standard deviation.
   a. 1_{2s} rule: The assay is "out of control" when a single control measurement exceeds the mean plus 2s or the mean minus 2s of previous control sample values, or it serves as a warning system to initiate additional inspection of control data. Variations in results that are caused by random error should have a Gaussian distribution (Figure 1.2). With that distribution, 95.45 % of the results should fall within the interval formed by the mean ± 2 SD. Therefore, if a QCM is analyzed 20 times, about 19 of 20 results are expected to be within that interval, but it is important to recognize that 1 of 20 results is expected to be outside of that interval, even when the assay is performing well!
   b. 1_{3s} rule: The assay is "out of control" when a single control measurement exceeds the mean plus 3s or the mean minus 3s of previous control sample values. This rule is primarily sensitive to random error.
   c. 2_{2s} rule: The assay is "out of control" when two consecutive control measurements exceed the mean plus 2s or the mean minus 2s of previous control sample values. This rule is primarily sensitive to systematic error.
   d. R_{4s} rule: The assay is "out of control" when one control measurement in a group exceeds the mean plus 2s and another exceeds the mean minus 2s of previous control sample values. This rule is primarily sensitive to random error.
   e. 4_{1s} rule: The assay is "out of control" when four consecutive control measurements exceed the mean plus 1s or the mean minus 1s of previous control sample values. This rule is primarily sensitive to systematic error.
   f. 10_x rule: The assay is "out of control" when 10 consecutive control measurements fall on one side of the mean of previous control sample values (this rule is sometimes modified to either an 8_x rule or a 12_x rule). This rule is primarily sensitive to systematic error.
4. Laboratory personnel can determine whether a random error or systematic error occurred based on which control rule was violated. With this information, the search for the cause of the error can be narrowed to the most likely analytical problems.
5. Selection of appropriate Westgard rules to control an assay depends on the amount of error inherent in the assay as well as the magnitude of change in a measurement that is considered to be biologically significant. An assay's inherent error is the TE_o (observed imprecision and inaccuracy) determined during assay validation or verification; in most clinical situations, RIs adjust for assay inaccuracies, so it is the degree of random error (imprecision) that is relevant. Biologically significant changes in an analyte are described by TE_a, a quality goal determined by a consensus of experts and defined as the greatest magnitude of variation in a result that does not alter clinical interpretation of the results.
   a. The TE_a for an assay depends on biological variations in health and magnitude of change in analyte measurements with disease. If intra-individual and inter-individual biological variations (see Analytical and Biological Variation, Section IV) of a particular analyte are small, and clinical decisions are made when there is only a minor change in a laboratory test result, then the random error of the assay needs to be relatively small. However, if clinical decisions are made only when there is a marked change in a laboratory test result, a larger random error might be acceptable. Two examples illustrate these concepts:

(1) Serum [Na^+] in most healthy mammals is near 150 mmol/L. If a patient has a serum [Na^+] of 150 mmol/L on day 1 and 160 mmol/L on day 2, most clinicians will conclude that there has been change for biological, pathologic, or therapeutic reasons. Therefore, for the serum Na^+ assay to be clinically valuable, its acceptable random error definitely needs to be < 10 mmol/L. Otherwise, erroneous decisions may be made because of random analytical error.

(2) Serum [glucose] in most healthy mammals is near 100 mg/dL. If a patient has a serum [glucose] of 100 mg/dL on day 1 and 110 mg/dL on day 2, most clinicians will conclude that such changes are within expected biological variation, i.e., there has not been a change for biological, pathologic, or therapeutic reasons. Therefore, for the serum glucose assay to be clinically valuable, its acceptable random error can be larger than the random error of the serum Na^+ assay.

b. The lesser the TE_o of an assay or the greater the TE_a, the less stringent the Westgard control rules need to be to help ensure reliable results. Tests done in most current chemistry analyzers perform with high precision and accuracy; therefore, less stringent Westgard rules, such as 1_{3s}, are sufficient to monitor performance and detect errors that indicate performance problems. Coagulation analyzers that employ manual pipetting of sample or reagent are more prone to analytical errors and thus require use of combined and more stringent Westgard rules that monitor both random and systematic error.[42]

6. Each Westgard rule or multirule (combination of rules), when applied to an assay with a predetermined TE_o, has a predictable probability of error detection (P_{ed}) and a predictable probability of false rejection (P_{fr}). The simplest rule or multirule that is capable of detecting errors ≥ 90 % of the time with ≤ 5 % false rejections is preferred. Performance of a rule or multirule can be increased by increasing the number of results (n) that are assessed in a "control run." An n of two can be achieved either by measuring one QCM twice or by measuring each of two QCMs once. Selecting QC rules that appropriately and efficiently monitor assay performance is called *QC validation*. The use of control charts (OPSpecs Charts) and software programs facilitate QC selection and validation (see www.westgard.com).[43,44]
7. A common method of monitoring the results of the control samples is to plot them in an electronic or paper Levey–Jennings control chart (Figure 1.5). This graphic display of serial QCM results facilitates assessment of Westgard rules. Prerequisites to using Levey–Jennings control charts include selection of a quality goal (TE_a), measurement of assay performance (TE_o), calculation of the mean and SD for each QCM, and validation of control rules.
8. Dr. Westgard has also addressed QA using concepts of total quality management and Six Sigma Quality Management (see www.westgard.com).[45] These concepts have been accepted by some international groups as the preferred QA method. The understanding and application of the Six Sigma Quality Management system requires considerable effort; a few key concepts introduce the topic:
   a. *Sigma* refers to the Greek letter σ, which stands for standard deviation, a statistical index used to express random deviation from the mean (central tendency).
   b. A *sigma* classification for a test refers to the inherent variability of its results relative to TE_a (includes random and systematic error), which is the greatest magnitude of variation in a result that does not alter clinical interpretation. The TE_a must be determined at specified clinical decision thresholds. For some analytes, critical clinical

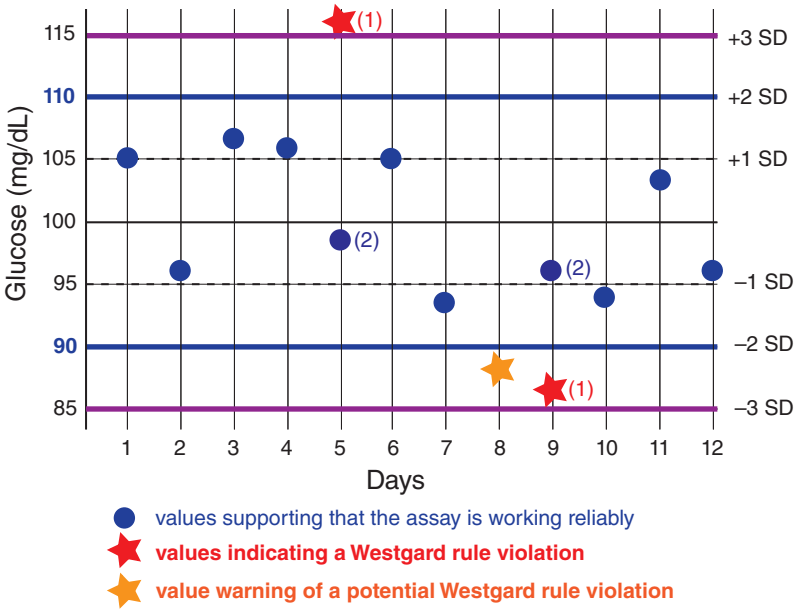


Figure 1.5. Levey–Jennings control chart for a glucose control solution. Quality assurance data for 12 d of testing are displayed; each day, the measured [glucose] in the QCM is plotted on the chart. Previous analyses established the mean (100 mg/dL) and SD (5 mg/dL) for the QCM's [glucose], allowing display of lines for ± 1, 2, and 3 SD.

- On day 5, the first measured concentration (1) was > 3 SD above the historical mean, thus violating the 1_{3s} Westgard rule and suggesting an analytical error. After corrective actions were taken to determine and correct the problem, a second measured concentration (2) was within acceptable limits, so values measured in subsequent patient samples could be considered reliable.
- On day 8, the QCM's [glucose] was more than 2 SD from the mean, representing a 1_{2s} warning that an error might be present, but this is expected to occur for 5 % of QCM results (1 in 20), so it could be a false alarm. In this case, the control value on day 9 was again > 2 SD below the mean, thus violating the 2_{2s} Westgard rule and suggesting an analytical error, likely systematic and not random. After corrective actions were taken, a second measured concentration (2) supported that the assay was again "in control."

For some assays, at least two control solutions are analyzed with each set of patient samples, and results of each control solution are plotted in separate Levey–Jennings control charts for each analyte. Control solutions are typically analyzed with each shift (e.g., day and night) or each day with the primary goal of detecting unacceptable assay performance before a patient's result is used for diagnostic or therapeutic decisions.

decisions are made at both increased and decreased quantities, so two TE_a values might be required.

c. Sigma is calculated as follows: $\sigma = (TE_a - |bias|) / CV_A$, where each value is expressed as a percentage to normalize σ over a wide range of quantities, and bias is expressed as the absolute value, so the direction of bias (positive or negative) is not a factor. In this equation, TE_a is a predetermined quality goal whereas bias and CV are determined during method-validation or verification studies (see Evaluating and Validating Laboratory Methods).

d. For theoretical assay A with a TE_a of 10 %, analysis of QCM with an analyte concentration near the decision threshold (100 mg/dL) reveals a SD of 5 mg/dL and

therefore a CV of 5 %. Assuming there is no systematic error (bias), this assay would be called a *two-sigma* assay because σ = 10 % / 5 % = 2 (and using concentrations instead of percentages, the TE_a divided by its SD is 2 also). When designing a QA program for this assay, there would be strict criteria established with several QCMs so that unacceptable errors could be detected when present. By random error alone, about 1 of 20 values is expected to be unacceptable.

e. For theoretical assay B, also with a TE_a of 10 %, analysis of QCM with an analyte concentration near the decision threshold (100 mg/dL) reveals a SD of 2 mg/dL and therefore a CV of 2 %. Assuming there is no systematic error (bias), this assay would be called a *five-sigma* assay because σ = 10 % / 2 % = 5 (and using concentrations instead of percentages, the TE_a divided by its SD is also 5). When designing a QA program for this assay, there would be relatively loose criteria established with two QCMs and one control rule (1_{3s}) because it is very unlikely that its random error would create changes that are clinically relevant (i.e., theoretically, less than one unacceptable value per million assays).

f. In the Six Sigma system, the ideal assay is a *six-sigma* assay because an unacceptable error would theoretically occur only 0.002 times per million assays (1 in 500 million). In a *six-sigma* assay, six standard deviations of variation are within the acceptable tolerance limits of the process.

VARIATIONS IN RESULTS ACROSS ASSAYS AND LABORATORIES

I. Not only do veterinarians face challenges of species variations, they also must deal with varied results generated by different laboratories and methods that are not all calibrated to a universal standard. There are many reasons for varied results; some of the differences are due to unpreventable random errors and others are caused by systematic errors (bias) identifiable and measurable by using method comparison studies.

II. Examples of variations

A. Table 1.10 and Figure 1.6 contain results of surveys completed by the Veterinary Laboratory Association (VLA) as part of an external QA program.[34] For such surveys, the VLA distributes aliquots of pooled samples of serum or whole blood to laboratories that subscribe to its QA program. After analysis of the distributed sample, each subscribing laboratory sends its results to the association for compilation and analysis. The association then distributes the results of the analyses. The type of program is sometimes referred to as a *proficiency testing* program and can be used to determine the validity of a laboratory's assay. Ideally, the results of an individual laboratory could be compared to the results established by a laboratory that analyzed the same sample by using a recognized reference method. Without such a value, the *proficiency testing* provides a method of periodically monitoring an assay's performance using *peer comparison*, the comparison of results to results of others who are using the same methods and types of analyzers.

B. Table 1.10 and Figure 1.6 illustrate marked differences in the results generated by laboratories, even for samples with few abnormalities (this dog's sample revealed only mild hypocalcemia, mildly increased alanine transaminase activity, and a 4.5× increase in alkaline phosphatase activity). Consequently, results from different assays or instruments within a laboratory or among laboratories (including multisite referral laboratories) should not be considered interchangeable unless this has been established by method comparison studies.

Table 1.10. Variation in serum chemistry results among labs using aliquots of one canine serum sample[a]

Analyte[b]	Number tested	Mean[c]	Unit	Low–high[c]	Range	Low to high as % change from mean	TE_a (%)[d]
Sodium	169	141	mmol/L	131–152	21	–7 to +8	5
Potassium	169	4.9	mmol/L	4.5–5.5	1.0	–8 to +12	10
Chloride	166	107	mmol/L	99–116	17	–7 to +8	5
tCa^{2+}	168	8.5	mg/dL	6.6–10.0	3.4	–22 to +18	10
Phosphorus	168	4.7	mg/dL	3.3–6.3	3.0	–30 to +34	20
tMg^{2+}	128	2.1	mg/dL	1.4–2.5	1.1	–33 to +19	20
Urea	165	21	mg/dL	17–29	12	–19 to +38	15
Creatinine	169	1.2	mg/dL	0.7–2.1	1.4	–42 to +75	20
Glucose	166	95	mg/dL	77–110	33	–19 to +16	20
Bilirubin, total	165	0.2	mg/dL	0.1–1.3	1.2	–50 to +550	30
Amylase	131	725	U/L	494–923	429	–32 to +27	25
ALP	164	459	U/L	311–1082	771	–32 to +136	25
CK	158	62	U/L	21–86	65	–66 to +39	30
AST	160	24	U/L	3–50	47	–88 to +108	30
ALT	164	83	U/L	52–112	60	–37 to +35	25
Total protein	165	6.1	g/dL	4.2–7.9	3.7	–31 to +30	10
Albumin	166	3.2	g/dL	2.8–4.1	1.3	–13 to +28	15
Cholesterol	162	264	mg/dL	221–294	73	–16 to +11	20

[a] Results from similar and different assay methods analyzed as part of one Veterinary Laboratory Association survey during 2022

[b] ALT, alanine transaminase; ALP, alkaline phosphatase; AST, aspartate transaminase; CK, creatine kinase; tMg^{2+}, total magnesium

[c] Mean values of all results for each analyte were provided in the survey report, while low and high values were extracted from graphs within the survey's report. All analyte values were rounded to reportable significant digits.

[d] TE_a, allowable total error, is the estimated error that is not expected to affect interpretation of results. These values are from TE_a guidelines of the American Society for Veterinary Clinical Pathology.[35] For each analyte, the difference between the lowest and highest values exceeds TE_a and could be clinically relevant if the values were obtained from the same animal over time. Similarly, for most analytes, the smaller changes from the lowest value to the mean and from the highest value to the mean could be considered clinically relevant, though three of each did not exceed the TE_a (see [tMg^{2+}], [glucose], [albumin], and [cholesterol]. Lack of agreement among laboratories emphasizes the need for establishment of reference intervals by each laboratory.

It is beyond the scope of this textbook to describe in detail the possible reasons for the differences; in general, results may vary because of one or more of the following:

1. Assay methods and instruments vary.
2. Commercial standard solutions vary across manufacturers and may be poorly calibrated against an appropriate reference. Although there are strict requirements for development of human assays, the same requirements for veterinary assays do not exist, in part because of species diversity.

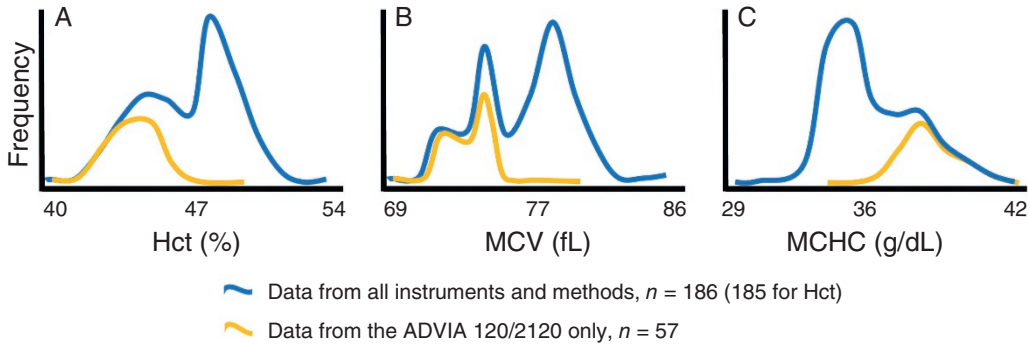


Figure 1.6. Interlaboratory variation in hematology results. These distribution curves reflect results from similar and different assay methods that analyzed aliquots of one canine whole blood sample as part of one survey completed by the Veterinary Laboratory Association (VLA) during 2022. These curves are close approximations of the histogram frequency distributions for Hct (A), MCV (B), and MCHC (C), three of the routine RBC analytes provided within the survey's report.

- *Blue curves*: These show the distribution of results for all participating laboratories, including all the different methods and analyzers used for these analytes. There is a wide variation in results. As for chemistry results of a similar survey in Table 1.10, the difference between the lowest and highest values would be considered clinically relevant if they represented values obtained from the same animal but different samples.
- *Gold curves*: These show the distribution for only those results generated by ADVIA analyzers. These results have narrower ranges than results from all laboratories because they were generated by the same methods and type of analyzer. Even for just this subset of results, the difference between the lowest and highest values would be considered clinically relevant if they represented values obtained from the same animal but different samples.
- Comparison of blue (all) and gold (ADVIA) curves also shows that the ADVIA results are lower (Hct and MCV) or higher (MCHC) than for other methods. The paired means for All and ADVIA results in this survey were, respectively: Hct, 47 % and 44 %; MCV, 77 fL and 73 fL; and MCHC, 35 g/dL and 38 g/dL.
- Lack of agreement among analyzers and methods necessitates establishment of RIs by each laboratory.

Hct, hematocrit; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume

3. For assays that measure enzyme activity or depend on enzymatic activity within the assay, substantial differences in results can occur because of assay variations that include differences in substrates (reagents), reaction temperatures, or pH of assay systems (see Chapter 12).
4. Even though two laboratories might have the same instrument and assay method, there is inherent variability in analyzers, and the laboratories might operate them in different environments (e.g., altitudes) and with different maintenance schedules, or purchase reagents from different companies. Even if the reagents have the same constituents, they might be at different concentrations.

C. Because of the potential for marked differences in results determined by different laboratories, several recommendations are made:
   1. Sample collection, processing, and transport should follow policies that minimize deterioration of sample quality.
   2. Samples should be submitted to veterinary laboratories that adhere to robust QA guidelines.
   3. A patient's results should be compared against appropriate RIs established for the same assay method in the same laboratory, when available. RIs established for another assay can be very misleading. Establishing de novo RIs and validation of transferred RIs from a manufacturer are described in the section titled Reference Intervals.

4. If comparing serial results across different analyzers, using the magnitude of change relative to the URL of each analyzer's RI may be more appropriate than directly comparing results. For example, if a dog with acute hepatic necrosis had a serum ALT activity of 1200 U/L (RI 75–120) at an emergency clinic and a second result of 600 U/L (RI 21–60) from a sample collected the next day at the patient's home clinic, the second result is half the first and might suggest improvement, but each is 10 times its respective URL, supporting that there is actually no change in hepatocellular damage. This presumes that both analyzers are performing well and that RIs were established appropriately.
5. A good diagnostician not only identifies but also confirms abnormalities that are at all questionable. If a laboratory result seems inconsistent with other findings for a case, a clinician should ask the laboratory to verify assay performance and, if possible, repeat the assay on the same sample and/or potentially use another analyzer that uses a different method.
6. Clinicians should establish strong professional relationships with their laboratory's personnel. A reputable laboratory strives to provide high-quality and timely results. As trusted healthcare providers, veterinarians should strive to provide their laboratories with high-quality samples.

WHICH LABORATORY SHOULD ONE USE?

I. Many factors must be considered before deciding how and where to obtain laboratory data for your patients.[46,47] Basically, there are three major options: (i) in-house laboratory, (ii) veterinary reference laboratory, and (iii) laboratory in local human hospital. Four major questions must be considered:
   A. How will the results affect the care of the patient?
   B. Can the laboratory consistently provide quality analysis of the sample? This includes access to expertise in veterinary clinical pathology.
   C. How much will it cost to analyze the sample?
   D. How important is a short turnaround time?

II. In-house laboratory (in a veterinary clinic or hospital)
   A. Advantages
      1. May allow 24-h access to laboratory data
      2. Shorter turnaround times, which are sometimes required for emergency cases (however, experts at outside laboratories may be particularly useful for sick patients)
      3. Fresh samples; thus fewer concerns about sample deterioration
      4. Client satisfaction because of fewer call-backs and repeat appointments (assuming no inaccurate results that complicate patient management)
      5. Potential for increased practice productivity, efficiency, and income
   B. Disadvantages
      1. Capital expenditure of thousands of dollars; equipment depreciates rapidly
      2. Need to maintain inventory of reagents and supplies
      3. Need sufficient personnel and meticulous training to proficiently operate and maintain equipment, recognize inappropriate samples, and process samples appropriately
      4. Need to follow a QA program and comply with any existing regulatory agency requirements to ensure high-quality results and minimize errors (Tables 1.7–1.9)
      5. Typically need high sample volume to generate enough income to cover expenses

6. RIs typically provided by the manufacturer of instruments or assay may or may not have been established appropriately or be relevant to the clinic's patient population or environment.
7. May need to ship a malfunctioning instrument to the manufacturer for repairs and thus need a replacement instrument or backup methods
8. Quality and value of the results are often inferior to those provided by veterinary reference laboratories, particularly for various microscopic assessments.

III. Veterinary reference laboratory
   A. Advantages
      1. Laboratory personnel are typically trained to provide quality analysis of veterinary samples and recognize samples that may lead to inaccurate results.
      2. Diagnostic support may be available from veterinary clinical pathologists in the laboratory, and veterinary specialists may be available for clinical consultations.
      3. RIs should be appropriate if derived from participating clinics' patient populations.
      4. Most laboratories have a quality management system in place to help ensure quality results. The system often includes proficiency testing by an external group such that performance can be compared to that of peers.
      5. Many more diagnostic assays are available than are available in an in-house laboratory.
      6. The cost for sample analysis is more clearly determined and thus can be charged to the client on a sample-by-sample basis.
      7. Some veterinary diagnostic companies manufacture in-clinic analyzers as well as operate reference laboratories with analyzers that allow higher throughput but that may employ methods and results similar to the in-clinic benchtop analyzers. Connectivity of in-house equipment with the manufacturer enhances monitoring of performance and troubleshooting.
   B. Disadvantages
      1. Not all analytes or samples are stable, and thus some deteriorate during shipment (e.g., urine, blood cells). Some analytes require special shipping, e.g., shipped frozen or in special tubes (collection tubes with added antiproteases).
      2. Turnaround times vary with location. Some are available the same day, and most are available the next day except for special tests.

IV. Laboratory in local human hospital
   A. An advantage is that turnaround times can be within hours.
   B. Disadvantages
      1. Quality RIs for veterinary samples may not be established by the laboratory.
      2. Assay methods may not be appropriate for veterinary samples. This is especially true for protein-based analytes (including enzymes and hormones) and immunologic-based assays.
      3. Technicians and technologists may not be trained to correctly identify species variations or diseases that are unique to veterinary samples.
      4. Pathologists who specialize in human patients might be very interested in providing consultation but lack training in the diseases of domestic animals and variations seen in veterinary samples.
      5. The fees charged by laboratories may be relatively high.
   C. Testing in local laboratories that specialize in human medicine should generally be avoided.

EVALUATING AND VALIDATING LABORATORY METHODS[24]

I. Reasons for evaluating laboratory methods
   A. New or revised laboratory methods may be evaluated for clinical use when the following are true: (i) recent research findings suggest a better approach, (ii) newer assays may be more accurate, more precise, more economical, or more efficient, or (iii) an instrument was purchased to replace an outdated or malfunctioning instrument.
   B. Before adopting new or revised methods, several questions should be asked. These include: Is the method practical? What are the equipment and space needs? Are personnel trained for the method? Will it improve patient care at a reasonable cost? Has there been adequate study of the method to prove its clinical value, or is the method still in the development stage? Are the requirements for sample collection, processing, and handling practical?

II. What are sources of analytical error?
   A. Imprecision: Each assay system has its own inherent random error causing imprecision (see Quality of Laboratory Results, Section II.B). Typically, the more precise the assay is, the better it is, but sometimes the most precise methods are too expensive or time consuming.
   B. Inaccuracy (bias): Besides random error, a method may not provide accurate results because of systematic error (bias). For example, the mean concentration of a new method may be consistently 5 mmol/L too high compared to the mean value determined by a reference method. In theory, *accuracy* is truth, but in reality, analytical accuracy is a relative term and sometimes difficult to assess in a clinical laboratory. An assay's result may be compared against one of two "true" values:
      1. The concentration determined by the reference method (a gold standard)
      2. The concentration determined by numerous analyses with comparative methods

III. Acceptable analytical performance, also called a quality goal
   A. Quality goals are often expressed as TE_a or the amount of error that does not change the interpretation and does not exceed the amount of error due to imprecision and biological variation. TE_a for an assay should be greater than TE_o, the amount of error measured (observed) in any assay.
   B. An assay does not have to be the most accurate, precise, specific, or sensitive assay to be clinically useful. It simply needs to fulfill predetermined requirements for acceptable performance.
   C. Criteria for acceptable performance of clinical assays have been proposed, and they vary considerably among analytes. A hematocrit method might be considered acceptable if it provides results within 10 % of the target value (e.g., 36–44 % for a hematocrit of 40 %). A creatinine method might be considered acceptable if creatinine concentrations are within 20 % of target values.[32,35]

IV. Analytical validation process
   A. Three stages of validation for analytical performance have been recommended with the goal of quantifying inherent analytical error (variation). This is in contrast to the validation of an assay's diagnostic performance described in the next section, a process pertaining to distinguishing between patients with and without a particular disorder. Detailed instructions for planning and carrying out rigorous method-validation studies have been published.[24]

B. Familiarization: This stage includes establishment of a working procedure, initial assessment of the analytical range, and calibration. Importantly, repetitive use serves as practice to "learn the assay," thereby promoting identification and reduction of errors and sources of variation.

C. Preliminary validation: In this stage, several studies are completed.
   1. Within-run precision (repeatability): to determine the degree to which promptly repeated analysis of the same samples yields the same results; if repeatability is poor, further validation may not be fruitful
   2. Linearity: to determine if signals (results) are proportional to analyte concentrations or activities, and over what analyte concentrations or activities they are linear and useful
   3. Interference studies: to determine the effects of common (e.g., hemolysis, bilirubin, lipemia) or potential relevant interferents; if clinically significant degrees of error (inaccuracy) are caused by clinically encountered concentrations of an interferent, results must be interpreted accordingly
   4. Recovery studies, if purified forms of the analyte are available: to determine if the assay is sufficiently accurate and can detect (recover) all the analyte added to samples (spiking) at relevant concentrations

D. Detailed validation: If initial results are satisfactory, then final validation includes the following components.
   1. Between-run precision (reproducibility) studies: to assess the reproducibility of results from a single sample tested over extended periods of time (e.g., 20 workdays, if the analyte is stable that long) to assess for imprecision caused by factors that vary over time (e.g., environment, operator)
   2. Comparison of methods with statistical analyses: to assess for accuracy compared to an accepted assay (see next section, Comparison of assays) in addition to, or instead of, a spiking and recovery study; patient specimens are the preferred samples
   3. Determination of acceptable performance criteria
   4. Establishment of RIs

E. Implementation phase
   1. If results of the validation procedures indicate that the assay is acceptable for use in the laboratory, then it needs to be incorporated into the daily routine of the laboratory, including equipment maintenance, reagent inventory, quality control procedures, and determination or validation of decision thresholds.
   2. When the assay is finally ready for clinical use, clinicians are notified of its availability, cost, and expected precision at the URL and LRL or major decision thresholds.

V. Comparison of assays: It is sometimes necessary to compare the results of two assays to determine the degree of analytical agreement between them, e.g., as part of validating a test by comparing it to a gold standard. Assays *agree* when they produce essentially the same results from the same samples and thus can be used interchangeably in making clinical decisions. Importantly, results may be strongly correlated but not in agreement. *Perfect correlation* means that the sample results for one assay always differ from those of another assay by the same percentage and direction; if results of one assay were always twice those of the other assay, the assays would correlate perfectly but they would not agree. The assays would have a *constant systematic bias* (difference between the results that is constant over all values). In contrast, if differences between assay results changed proportionately with the magnitude of the result, there would be a *proportional systematic bias*. Methods of assessing assay agreement and guidelines for completing and interpreting method comparison studies have been reviewed.[48,49] Veterinarians can benefit from awareness of what information the four major methods provide.

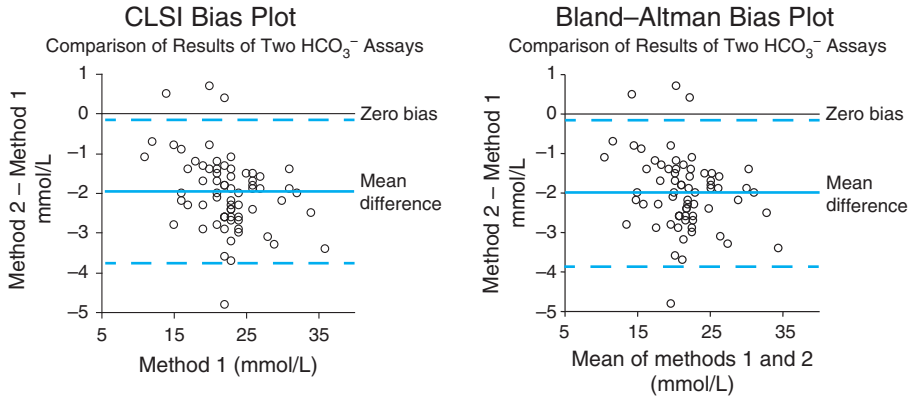


Figure 1.7. CLSI and Bland–Altman bias plots. The results were tabulated and entered in a software program (Analyse-it) to generate the bias plots.

- In the CLSI bias plot, the differences between HCO_3^- concentrations measured by two assays (method 2 – method 1 in this example) are plotted on the *y*-axis; the concentrations measured by method 1 are plotted on the *x*-axis. The mean difference for all samples is calculated. In this comparison, the mean difference is 2 mmol/L, so method 2 has a bias of –2 mmol/L compared to method 1. Lines representing ± 1.96 SD of the differences are also displayed as *limits of agreement*, the boundary for 95 % of the differences. The CLSI bias plot is the preferred plot when a second or test method is being compared to a reference method or an established (comparative) method.
- The Bland–Altman bias plot is the same as the CLSI bias plot except the average of the two measured values (average of method 1 and method 2 concentrations) are plotted on the x-axis. The Bland–Altman bias plot is used when neither method is considered to be more accurate.
- When results show a bias, a relatively flat distribution indicates a constant bias and a sloped distribution supports a proportional bias (this distribution tends to run from upper left to lower right, but more data would be needed to be sure). Determining if the bias is tolerable and manageable involves consideration of a quality goal for the assay (e.g., TEa), the number of results outside the limits of agreement, and the type of bias (proportional or constant).

HCO_3^-, bicarbonate

Choice of method is beyond the scope of this book, but depends on the type of result (continuous, discrete, ordinal), the range of data, the degree of correlation, and the type of any proportional or constant bias that is present.

A. Bias plots (Figure 1.7) for continuous data of narrow or wide range are particularly useful for analytes with a narrow range of data (e.g., electrolytes) and a correlation coefficient $r < 0.95$.
   1. CLSI bias plot (Note: CLSI was previously NCCLS, National Committee for Clinical Laboratory Standards, so this was called a NCCLS bias plot.)
      a. The CLSI plot displays the degree of agreement between paired values generated by a new method and the old method (or reference method). The values determined by the old/reference method are plotted on the *x*-axis, and the difference between values determined by the new and old method is plotted on the *y*-axis. Besides plotting the paired data points, the mean and SD of each difference is also plotted.
      b. The graph displays a positive bias (mean difference > 0) or a negative bias (mean difference < 0) and may indicate a *constant bias* (the plotted differences form a flat line significantly above or below the line of zero bias), a *proportional bias* (differences form a straight line that slopes up or down significantly compared to the line of zero bias), or both biases.

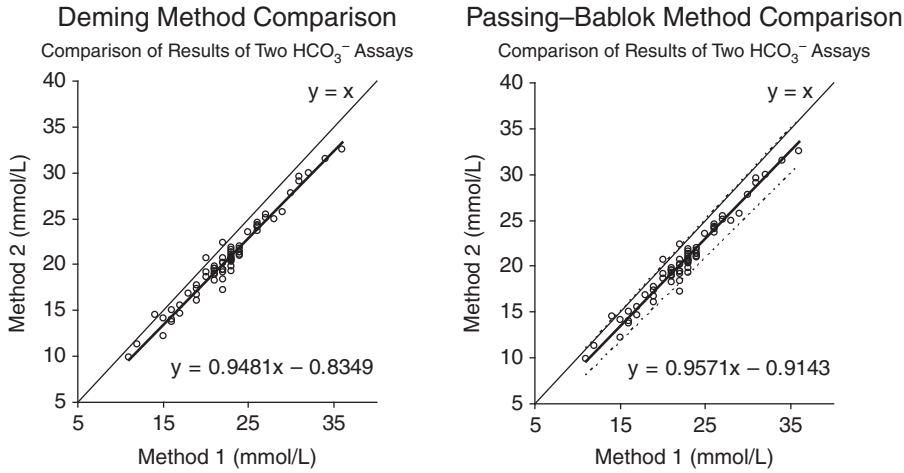


Figure 1.8. Deming and Passing–Bablok method comparisons. The results were tabulated and entered in a software program (Analyse-it) to generate the comparison graphs. The analysis of the two graphs reveals a mild proportional bias, i.e., the difference between results for the two methods increases at higher concentrations.

- In the Deming method, the results of the two assays are plotted on the *y*-axis and the *x*-axis, and a best-fit regression line is drawn and compared to the identity line ($y = x$). The equation for the best-fit line ($y = mx + b$) and associated confidence intervals are used to detect constant bias or proportional bias. In this method of comparison, imprecision of the assays should be normally distributed.
- The Passing–Bablok method comparison is very similar to the Deming method, but the imprecision need not be normally distributed and can have nonconstant variance over the sampling range. The 95 % confidence interval of the best-fit line is also displayed (dashed lines).

HCO_3^-, bicarbonate

2. The Bland–Altman bias plot is similar but with the following difference: the average of the two values is plotted on the *x*-axis and the difference between the two values is plotted on the *y*-axis. This method does not assume that either the new or the old method is more accurate.

B. Deming method comparison (Figure 1.8) for continuous data with wide range (e.g., many enzyme activities)
   1. In the Deming method comparison, the values obtained for a given sample are plotted with the new method on the *y*-axis and the old (or comparison) method on the *x*-axis. A regression equation for the best-fit line ($y = mx + b$) and the 95 % confidence intervals for the slope and *y*-intercepts are calculated.
      a. A *proportional bias* is detected if the 95 % confidence interval for the slope does not include 1 (i.e., slope of the identity line).
      b. A *constant bias* is detected if the 95 % confidence interval for the *y*-intercept does not include 0 (i.e., *y*-intercept of the identity line).
      c. Perfect agreement would yield a slope of 1 and a *y*-intercept of 0.
   2. The Deming method allows comparison of two independent variables which in this case are test methods, each with imprecision. As with any assessment of agreement, the comparison is improved with duplicate measurements by both methods because this reduces the effect of measurement imprecision.

3. In contrast to Deming regression, simple linear regression assumes that the x value is an independent variable with no error and the y value is dependent on x. A correlation coefficient (r) is computed to assess the strength and direction of the linear relationship between the two variables; $r = 0$ when there is no correlation and $r = 1$ when the correlation is perfect. A strong positive linear relationship is expected if two assays are designed to measure the same analyte concentration.
   a. Although method comparison studies involve *two independent* variables, linear regression statistics are used to assess agreement when errors in a reference method are small and the correlation coefficient is very high (e.g., $r > 0.99$).
   b. The correlation coefficient is very dependent on the range of data in the sample.[50, 51] A wide range of data tends to have a higher correlation than a narrow range. This is true even when most values are clustered and a single value stretches the range more widely. If such a value is aberrant, it can erroneously affect the relationship, suggesting more or less of a relationship than actually exists. Data sets should be broad and without large gaps between values.
   c. Figures 1.7 and 1.8 depict data with excellent correlation ($r = 0.98$) between data sets, but the bias plots illustrate there is a bias between the assays.

C. Passing–Bablok method comparison (Figure 1.8) for continuous data with a wide range
   1. The Passing–Bablok method is similar to the Deming method, but it is a nonparametric approach that does not require normally distributed measurement errors.
   2. This method estimates a linear regression line and tests whether the intercept is 0 and the slope is 1 as would be true of a line of identity.

D. Cohen's kappa test can be used to assess the agreement of two assays that generate results on ordinal scales, e.g., 1+, 2+, 3+, and 4+, or trace, mild, moderate, and marked. Details and caveats are beyond the scope of this book.
   1. The calculated kappa value provides a guide to the degree of agreement. Generally, agreement is considered poor with a kappa < 0.2, fair with kappa ≥ 0.2 and < 0.4, moderate with kappa ≥ 0.4 and < 0.6, good with kappa ≥ 0.6 and < 0.8, and very good with kappa of 0.8–1.0.
   2. The size or degree of disagreement is not considered with the standard kappa method, but it is with the weighted kappa method.

E. Interpretation of assay comparison studies
   1. After the quantitative aspects of the assays are compared, a decision is made as to whether the degree of agreement is acceptable such that the methods can be used interchangeably. Acceptability should be based on a performance goal, often TE_a, defined prior to the study.
   2. Visual inspection of graphed data may reveal that the results differ sufficiently so that the two assays cannot be considered the same. Thus, there would need to be different RIs and decision thresholds for the two assays.
   3. What would be the results of a comparison study if the new assay is actually better than the existing gold standard? The new assay could appear to perform poorly and might be rejected when it would actually be an improvement. This scenario occurs as better new tests are sought to replace poorer old tests. To validate accuracy of a new test in such a situation, spiking and recovery of purified analyte may be more useful than a method comparison.
   4. Because no assay is perfectly precise, differences between assays are expected even if they have excellent agreement. The amount of inherent difference can be assessed by calculating a combined inherent CV.[48]
   5. The acceptability of a new assay can also be assessed using a medical decision chart.[48]

F. Error grid analysis provides a visual assessment of agreement and is a nonparametric graphical approach for comparing the potential harm caused by differences in results between two measurement methods.[52] After acquiring approximately 100 paired measurements or more, data are plotted on a graph with the reference method measurement on the *x*-axis and the new method on the *y*-axis as for regression analysis. A so-called error grid is applied to the graph to denote three zones: A – the desirable region near the line of identity ($x = y$) that denotes no risk to the patient and therefore acceptable total error of the new test relative to the existing test, B – an intermediate zone outside of zone A, and C – a zone of unacceptably high errors likely to cause patient harm. For an appropriate replacement test, the vast majority of results should fall within zone A, a small proportion in zone B, and very few in zone C.

DIAGNOSTIC PROPERTIES AND PREDICTIVE VALUE OF LABORATORY ASSAYS

I. After ensuring that the analytical performance of an assay is acceptable for clinical use, valid RIs can be used to identify atypical results, but several disorders might be responsible for an increased or decreased result. If an assay is to be used to identify a particular disorder, the assay's diagnostic performance should be assessed to determine if the assay can distinguish between patients that have a disorder (disease positive) and patients that do not.[49] In so doing, it is important that the group of patients without the disorder includes patients with similar clinical findings and disorders that must be differentiated from the disorder of interest; if it includes only healthy patients, the observed diagnostic performance will be falsely optimistic and not reflective or predictive of actual performance in a clinical setting.

II. There are four classifications of test results relative to the presence or absence of a disease:
   A. TP (true positive): a result that correctly identified a patient as having a specified disease
   B. TN (true negative): a result that correctly identified a patient as not having a specified disease
   C. FP (false positive): a result that incorrectly identified a patient as having a specified disease
   D. FN (false negative): a result that incorrectly identified a patient as not having a specified disease

III. To classify test results into one of the four categories, two factors must be known:
   A. What is the diagnostic threshold (clinical decision limit) used to discriminate between individuals with the disease of interest (disease positive) and individuals with other disorders or no disorder (disease negative)?
      1. Is the decision limit either an upper or lower reference limit? Although RIs describe the expected location and spread of values in health, they generally reflect only the central 95 % of healthy individuals and are typically not useful to identify any particular disorder.[19]
      2. Or is the clinical decision limit within or outside of the RI? Clinical decision limits may be empirically determined (see ROC section) and applied, e.g., for pancreatic lipase immunoreactivity to diagnose canine pancreatitis, or for calf serum [IgG] to assess for transfer of passive immunity.
   B. What determines whether an animal has the disease of interest? That is, what is the gold standard that allows us to say that the animal definitely does or does not have the disease? For many spontaneous diseases, there may not be a gold standard, either a single test or specific constellation of findings. Diagnosis might be based on investigators' own criteria or consensus criteria from experts, which might require a preponderance of evidence from various tests, long-term follow-up, or even necropsy. Conclusions about the diagnostic value of a test must be interpreted relative to the quality of the selected diagnostic criteria.

IV. After test results are appropriately classified as TP, TN, FP, or FN, several calculations are made in an attempt to characterize the diagnostic properties or predictive value of the assay. It is important to differentiate diagnostic performance (e.g., diagnostic sensitivity, specificity, and accuracy), as discussed here, from analytical performance (analytical sensitivity, specificity, and accuracy), as discussed in the section on Quality of Laboratory Results (II.C, D, and F.)[49]

A. *Diagnostic sensitivity*

1. Definition: the frequency with which a test is positive in patients that have the disorder of interest (Eq. 1.2a)

In the following equations, multiply results by 100 to convert from decimals to percentages (e.g., 0.8 × 100 = 80 %); # stands for number.

$$\text{Diagnostic sensitivity} = \frac{\#\text{ of true positive}}{\#\text{ with specified disease}} = \frac{\text{TP }\#}{\text{TP }\# + \text{FN }\#} \quad (\mathbf{1.2a})$$

$$\text{Diagnostic specificity} = \frac{\#\text{ of true negative}}{\#\text{ without specified disease}} = \frac{\text{TN }\#}{\text{TN }\# + \text{FP }\#} \quad (\mathbf{1.2b})$$

$$\text{Diagnostic accuracy} = \frac{\#\text{ correctly classified}}{\#\text{ of animals in study}} = \frac{\text{TP }\# + \text{TN }\#}{\text{TP }\# + \text{FP }\# + \text{TN }\# + \text{FN }\#} \quad (\mathbf{1.2c})$$

$$\text{PPV} = \frac{\#\text{ of true positive}}{\text{all positive results}} = \frac{\text{TP }\#}{\text{TP }\# + \text{FP }\#} \quad (\mathbf{1.2d})$$

$$\text{NPV} = \frac{\#\text{ of true negative}}{\text{all negative results}} = \frac{\text{TN }\#}{\text{TN }\# + \text{FN }\#} \quad (\mathbf{1.2e})$$

$$\text{PPV} = \frac{\text{sensitivity} \times \text{prevalence}}{(\text{sensitivity} \times \text{prevalence}) + [(1 - \text{specificity}) \times (1 - \text{prevalence})]} \quad (\mathbf{1.2f})$$

$$\text{NPV} = \frac{\text{specificity} \times (1 - \text{prevalence})}{[(1 - \text{sensitivity}) \times \text{prevalence})] + [\text{specificity} \times (1 - \text{prevalence})]} \quad (\mathbf{1.2g})$$

2. A test that has high diagnostic sensitivity is a good one for screening for the presence of a disease because the test has very few FN results. If the animal has the disease, there is a high probability that the test will be positive. Conversely, if the test is negative, it is likely that the animal does not have the disorder, although this depends on other factors, including the prevalence of the disorder in the population.
3. A mnemonic for the concept of a high-sensitivity test helping to *rule out* a disorder is SnNOut (high **Sn**sitivity **N**egative rules disease **Out**).[53]

B. *Diagnostic specificity*

1. Definition: the frequency with which a test is negative in patients that do not have the disorder of interest (Eq. 1.2b)

2. A test that has high diagnostic specificity can be a good one for confirming (ruling in) that an animal has a disorder because the test has very few FP results. If the result is positive, there is a high probability that the animal will have the disorder, although this depends on other factors, including the prevalence of the disorder in the population. A mnemonic for the concept of a high-specificity test helping to rule in a disorder is SpPIn (high **Sp**ecificity **P**ositive rules disease **In**).[53]

C. *Diagnostic accuracy*
1. Definition: the frequency with which a test correctly classifies an animal as having or not having the disorder (Eq. 1.2c)
2. A test has high diagnostic accuracy when it has relatively few FP and FN results compared to TP and TN results.

D. *Positive predictive value (PPV)*, the predictive value of a positive test
1. Definition: the probability that a positive test result indicates that the animal has the disease (Eq. 1.2d)
2. A test that has a high PPV is one that has very few FP compared to TP results. Thus, a positive test result strongly suggests the presence of the disease.

E. *Negative predictive value (NPV)*, the predictive value of a negative test
1. Definition: the probability that a negative test result indicates that the animal does not have the disease (Eq. 1.2e)
2. A test that has a high NPV is one that has very few FN compared to TN results. Thus, a negative test result strongly suggests the absence of the disease.

V. Basic concepts of predictive values: Three major questions are considered when the diagnostic properties of an assay are evaluated.
A. What criteria are used to establish the presence or absence of disease? Definitive diagnoses are sometimes very difficult to reach for spontaneous diseases, and errors in disease state classification affect predictive value results.
B. What is the decision threshold chosen to indicate a particular disorder is present? Extensive evaluation of an assay in a carefully planned study is needed to find the best clinical decision limit, and this should be done prior to assessment of diagnostic performance. The effects of changing the decision threshold on diagnostic performance are illustrated in Figure 1.9. Different decision thresholds may be used for making different clinical decisions for the same disorder or for the same analyte for different disorders.
C. What is the prevalence of the disease in the studied population? The effect of prevalence is illustrated in Figure 1.10. Basically, when the disease prevalence is very low, there are relatively few TP results and a relatively greater percentage of FP results, so the PPV will be lower. Conversely, when the disease prevalence is very high, it is more likely that there will be FN results; the NPV will be lower and the PPV will be higher compared to a population with a lower prevalence of the disease.[54] Unlike predictive values, diagnostic sensitivity and specificity are calculated based only on test and disease positivity, and test and disease negativity, respectively; therefore, prevalence does not affect them.

VI. Application and interpretation of predictive value concepts
A. The clinical value of the calculated diagnostic properties is influenced by the prevalence of a disease. For examples A and B in Figure 1.10, assume the diagnostic sensitivity of a test is 90 % and its diagnostic specificity is 80 %.
1. In example A of Figure 1.10, with a disease prevalence of 30 %:
a. The PPV is 66 %; for all positive test results, 66 % are TP results.
b. The NPV is 95 %; for all negative test results, 95 % are TN results.

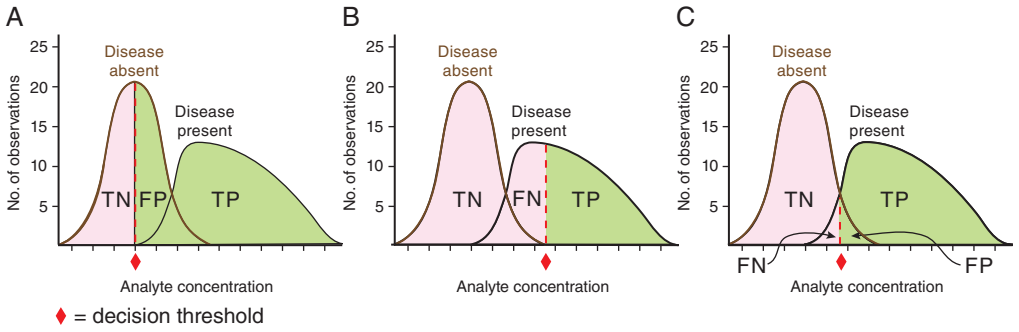


Figure 1.9. Effects of different decision thresholds on classifying test results. In these examples, the distribution of observed values in the animals without the disease appears to be Gaussian; such a distribution may or may not be true in real studies. The distribution of data in the diseased group is not Gaussian; typically, such data are not Gaussian but may not be skewed as shown in this example.

A. The decision threshold is near the mean analyte concentration found in the animals without the disease. With such a decision threshold:
   - The diagnostic sensitivity is 100 % because there are no FN results.
   - The diagnostic specificity is 50 % because there are equal numbers of TN and FP results.
   - The diagnostic accuracy is poor because of the many FP results.
   - The PPV is poor because of the many FP results
   - The NPV is 100 % because there are no FN results.

B. The decision threshold is at the highest value found in the animals without the disease.
   - The diagnostic sensitivity is poor (~ 60 %) because there are relatively many FN results.
   - The diagnostic specificity is 100 % because there are no FP results.
   - The diagnostic accuracy is poor because of the many FN results.
   - The PPV is 100 % because there are no FP results.
   - The NPV is poor because there are many FN results.

C. The decision threshold is at a concentration with the least overlap between the groups.
   - This decision threshold represents a compromise to obtain the best combination of diagnostic sensitivity and diagnostic specificity, and provides the best diagnostic accuracy because there are relatively few FP and FN results.
   - The values for PPV and NPV are high but not 100 %.

2. In example B of Figure 1.10, with a disease prevalence of 1 %:
   a. The PPV is 4.3 %; for all positive test results, 4.3 % are TP results.
   b. The NPV is 99.9 %; for all negative test results, 99.9 % are TN results.
3. By comparing the results of the two examples, these conclusions are formed:
   a. As the prevalence of the disease dropped from 30 % to 1 %, the PPV decreased from 66 % to 4.3 %. Thus, the PPV is less when the prevalence of the disease is lower.
   b. As the prevalence of the disease dropped from 30 % to 1 %, the NPV increased from 95 % to 99.9 %. Thus, the NPV is greater when the prevalence of the disease is lower.

B. The predictive value concepts are used to compare the diagnostic value of two laboratory tests. To illustrate this application, data were extracted from an article that compared the diagnostic value of serum $[tT_4]$ and $[fT_4]_{ed}$ for diagnosing feline hyperthyroidism.[55] As described in Chapter 17, serum $[tT_4]$ and $[fT_4]_{ed}$ may increase in sera of cats with hyperthyroidism, but other factors can influence $[tT_4]$ and $[fT_4]_{ed}$.

Example A – 30 % disease prevalence

Step 1	Disease present		Disease absent	
Positive test	(TP)	270	(FP)	140
Negative test	(FN)	30	(TN)	560
Totals		300		700

Step 2	Disease present		Disease absent		Totals
Positive test	(TP)	270	(FP)	140	410
Negative test	(FN)	30	(TN)	560	590
Totals		300		700	1000

Step 3

$$\text{Predictive value of positive test (as \%)} = \frac{\text{TP \#}}{\text{TP \# + FP \#}} \times 100 = \frac{270}{410} \times 100 = 66\ \%$$

$$\text{Predictive value of negative test (as \%)} = \frac{\text{TN \#}}{\text{TN \# + FN \#}} \times 100 = \frac{560}{590} \times 100 = 95\ \%$$

Example B – 1 % disease prevalence

Step 1	Disease present		Disease absent	
Positive test	(TP)	9	(FP)	198
Negative test	(FN)	1	(TN)	792
Totals		10		990

Step 2	Disease present		Disease absent		Totals
Positive test	(TP)	9	(FP)	198	207
Negative test	(FN)	1	(TN)	792	793
Totals		10		990	1000

Step 3

$$\text{Predictive value of positive test (as \%)} = \frac{\text{TP \#}}{\text{TP \# + FP \#}} \times 100 = \frac{9}{207} \times 100 = 4.3\ \%$$

$$\text{Predictive value of negative test (as \%)} = \frac{\text{TN \#}}{\text{TN \# + FN \#}} \times 100 = \frac{792}{793} \times 100 = 99.9\ \%$$

Figure 1.10. Examples of diagnostic properties of assays. These examples demonstrate the effect of prevalence on predictive values. For each example, the values for diagnostic sensitivity and specificity are 90 % and 80 %, respectively.

- *In example A*, we discovered (via a gold standard) that 30 % of 1000 dogs have the disease. Based on the prevalence, what is the test's positive predictive value? What is the test's negative predictive value?
  - *Step 1*: Construct a table from the available information. Because 30 % of the dogs have the disease, 300 dogs have the disease and 700 dogs do not. Given the diagnostic sensitivity of 90 %, 90 % (270) of the 300 diseased dogs will have a positive test result (TP) and 30 will have a negative test result (FN). Because the diagnostic specificity is 80 %, 80 % (560) of 700 dogs will have negative results (TN) and 140 will have a positive result (FP).
  - *Step 2*: Add the values for the number of positive and negative results.
  - *Step 3*: Calculate the positive predictive value and negative predictive value by using Eqs. 1.2d and e.
- *In example B,* we discovered (via a gold standard) that 1 % of 1000 dogs have the disease. Based on the prevalence, what is the test's positive predictive value? What is the test's negative predictive value?
  - *Step 1*: Construct a table from the available information. Because 1 % of the dogs have the disease, 10 dogs have the disease and 990 dogs do not. Given the diagnostic sensitivity of 90 %, 90 % (9) of the 10 diseased dogs will have a positive test result (TP) and 10 % (1) will have a negative test result (FN). Because the diagnostic specificity is 80 %, 80 % (792) of 990 dogs will have negative results (TN) and 198 will have a positive result (FP).
  - *Step 2*: Add the values for the number of positive and negative results.
  - *Step 3*: Calculate the positive predictive value and negative predictive value by using Eqs. 1.2d and e.
- When prevalence and diagnostic sensitivity/specificity data are known, positive and negative predictive values can be calculated from Eq. 1.2f and 1.2g. Plugging in different prevalence values to those equations demonstrates the effect of prevalence on predictive values.

1. Gold standard for this study
   a. Cats were classified as having hyperthyroidism by using the following clinical or laboratory findings:
      (1) Clinical signs consistent with hyperthyroidism
      (2) Palpable thyroid nodule
      (3) Good clinical response to treatment for hyperthyroidism
      (4) Basal [tT_4] increased or basal [tT_4] not increased, but positive results of triiodothyronine-suppression or thyrotropin-releasing hormone stimulation test
   b. Cats were classified as not having hyperthyroidism if they did not meet hyperthyroidism criteria. These cats had clinical signs suggestive of hyperthyroidism (e.g., weight loss, vomiting, diarrhea, and polyuria), but none had a palpable thyroid mass, none had increased [tT_4], and all had a diagnosis other than hyperthyroidism. Note that healthy individuals were not included, as is appropriate for studies of diagnostic accuracy involving tests intended to distinguish a specific disorder, in this case hyperthyroidism, from other disorders with similar clinical findings. Inclusion or use only of healthy subjects can inappropriately augment assay diagnostic performance.
2. Classification of results
   a. tT_4 results were classified as positive if [tT_4] was > 48 nmol/L. fT_4 results were classified as positive if $[fT_4]_{ed}$ was > 51 pmol/L. Decision thresholds represented the URLs determined from 172 healthy cats.
   b. Results were classified as negative if they did not meet positive criteria.
3. Based on these criteria for the 1138 cats:
   a. 917 cats had hyperthyroidism. Of these, 837 had an increased [tT_4] and 903 had increased $[fT_4]_{ed}$.
   b. 221 cats did not have hyperthyroidism. Of these, none had increased [tT_4] and 14 had increased $[fT_4]_{ed}$.
4. From the data provided, tables were constructed to show the classification of test results. From the tabulated data, the diagnostic properties and predictive values of [tT_4] and $[fT_4]_{ed}$ were calculated (Figure 1.11).
5. Based on the evaluation of reported data and application of the aforementioned gold standard and decision thresholds, these conclusions can be drawn:
   a. Serum $[fT_4]_{ed}$ had better diagnostic sensitivity (98 %) than did serum [tT_4] (91 %) for detecting feline hyperthyroidism. Thus, serum $[fT_4]_{ed}$ would be a better screening test for hyperthyroidism, i.e., more cats with hyperthyroidism have increased $[fT_4]_{ed}$ than increased [tT_4].
   b. Serum [tT_4] had better diagnostic specificity (100 %) than did serum $[fT4]_{ed}$ (94 %), i.e., [tT_4] had fewer FP results. Thus, an increased serum [tT_4] is more indicative of feline hyperthyroidism than is an increased serum $[fT_4]_{ed}$.
   c. Serum $[fT_4]_{ed}$ had better diagnostic accuracy (98 %) than did serum [tT_4] (93 %). Thus, if only [tT_4] or $[fT_4]_{ed}$ can be determined, serum $[fT_4]_{ed}$ has a better chance of correctly classifying the cat as having or not having hyperthyroidism. However, measuring $[fT_4]_{ed}$ is more expensive than measuring [tT_4].
   d. The PPV for increased [tT_4] was 100 %, and it was 98 % for $[fT_4]_{ed}$.
   e. The NPVs for [tT_4] and for $[fT_4]_{ed}$ were 73 % and 94 %, respectively. These results indicate that a $[fT_4]_{ed}$ that is WRI would strongly suggest that a cat does not have hyperthyroidism because there were very few FN results.

$[tT_4]$ in feline sera

	Hyperthyroidism present		Hyperthyroidism absent		Totals
Positive test	(TP)	837	(FP)	0	837
Negative test	(FN)	80	(TN)	221	301
Totals		917		221	1138

$$\text{Diagnostic sensitivity (as \%)} = \frac{\text{TP \#}}{\text{TP \#} + \text{FN \#}} \times 100 = \frac{837}{917} \times 100 = 91\ \%$$

$$\text{Diagnostic specificity (as \%)} = \frac{\text{TN \#}}{\text{TN \#} + \text{FP \#}} \times 100 = \frac{221}{221} \times 100 = 100\ \%$$

$$\text{Diagnostic accuracy (as \%)} = \frac{\text{TP \#} + \text{TN \#}}{\text{TP \#} + \text{FP \#} + \text{TN \#} + \text{FN \#}} \times 100 = \frac{1058}{1138} \times 100 = 93\ \%$$

$$\text{Predictive value of positive test (as \%)} = \frac{\text{TP \#}}{\text{TP \#} + \text{FP \#}} \times 100 = \frac{837}{837} \times 100 = 100\ \%$$

$$\text{Predictive value of negative test (as \%)} = \frac{\text{TN \#}}{\text{TN \#} + \text{FN \#}} \times 100 = \frac{221}{301} \times 100 = 73\ \%$$

$[fT_4]_{ed}$ in feline sera

	Hyperthyroidism present		Hyperthyroidism absent		Totals
Positive test	(TP)	903	(FP)	14	917
Negative test	(FN)	14	(TN)	207	221
Totals		917		221	1138

$$\text{Diagnostic sensitivity (as \%)} = \frac{\text{TP \#}}{\text{TP \#} + \text{FN \#}} \times 100 = \frac{903}{917} \times 100 = 98\ \%$$

$$\text{Diagnostic specificity (as \%)} = \frac{\text{TN \#}}{\text{TN \#} + \text{FP \#}} \times 100 = \frac{207}{221} \times 100 = 94\ \%$$

$$\text{Diagnostic accuracy (as \%)} = \frac{\text{TP \#} + \text{TN \#}}{\text{TP \#} + \text{FP \#} + \text{TN \#} + \text{FN \#}} \times 100 = \frac{1110}{1138} \times 100 = 98\ \%$$

$$\text{Predictive value of positive test (as \%)} = \frac{\text{TP \#}}{\text{TP \#} + \text{FP \#}} \times 100 = \frac{903}{917} \times 100 = 98\ \%$$

$$\text{Predictive value of negative test (as \%)} = \frac{\text{TN \#}}{\text{TN \#} + \text{FN \#}} \times 100 = \frac{207}{221} \times 100 = 94\ \%$$

Figure 1.11. Analysis of the diagnostic properties of serum $[tT_4]$ and $[fT_4]_{ed}$. The source of the data for this figure is explained in the text. Conclusions are also presented in the text.

- *[tT4] data*
  - From the data provided, a table was constructed to show the classification of test results.
  - From the tabulated data, the diagnostic properties and predictive values of serum $[tT_4]$ were calculated.
- *$[fT_4]_{ed}$ data*: the same procedures were completed.

f. Note that the results of such studies may vary with different assays, different populations, different gold standards, and different decision thresholds.
   (1) $[tT_4]$ was used to determine the presence or absence of hyperthyroidism. The gold standard used (or method of establishing the presence of hyperthyroidism) in the study is considered an excellent method of establishing the presence of feline hyperthyroidism, but it would have been interesting to learn whether the diagnostic value data for $[tT_4]$ would change if $[tT_4]$ had not been used to help determine the presence or absence of hyperthyroidism.
   (2) Most cats in the study had been referred to specialists for treatment of hyperthyroidism. Thus, there was a high prevalence of hyperthyroidism in the study's population. A high prevalence increases the PPV of diagnostic tests and lowers the NPV. Accordingly, the predictive values of $[tT_4]$ and $[fT_4]_{ed}$ would probably be different in a nonreferral veterinary practice.

VII. Application of the aforementioned methods to evaluate and compare diagnostic methods requires careful planning, appropriate choices of disease-present and disease-absent populations, and availability of an excellent gold standard.[56]
   A. A deficiency in the diagnostic properties and predictive value theories is that a positive result is given the same weight or importance if the value is only slightly increased or if it is extremely increased. In practice, the degree to which a result is abnormal does affect clinical decisions.
   B. After a diagnostic test (assay) has been thoroughly studied in a specific clinical context, findings regarding its diagnostic performance provide guidance for its expected performance in other laboratories with different patient populations and clinicians. However, six criteria should be fulfilled before the diagnostic properties (predictive value, diagnostic accuracy, etc.) can be considered completely transferrable to another clinical setting:[57]
      1. The definition of the disease is constant, and the diagnostic criteria are applied in the same way such that two animals with the same abnormalities in two different settings would be diagnosed with the same disorder.
      2. The same test is used. However, as described in earlier sections, complete agreement between two analytical assays may not be present even if the same instrument and reagents are used.
      3. The decision thresholds between categories of test results are constant and clear, i.e., two different observers should agree on results that are *positive* and on results that are *negative*.
      4. The distribution of test results in the disease group is constant, i.e., the data should have the same average and same distribution curve. However, as for RIs, decision limits are specific for the demographics of the population in which they are determined, so it may be difficult to fulfill this criterion. The demographics of the patient population in a location to which a decision limit is transferred might not fully replicate the original patient population in terms of severity or stage of disease. Data for animals with mild disease probably differ from those with severe disease.
      5. The distribution of test results in the disease-absent group is constant, i.e., the data should have the same average and same distribution curve. It may be difficult to fulfill this criterion. Certain disorders may be rare in a primary-care clinic, yielding more FP results, but very common in a referral clinic, resulting in more FN results, a lower NPV, and a higher PPV.

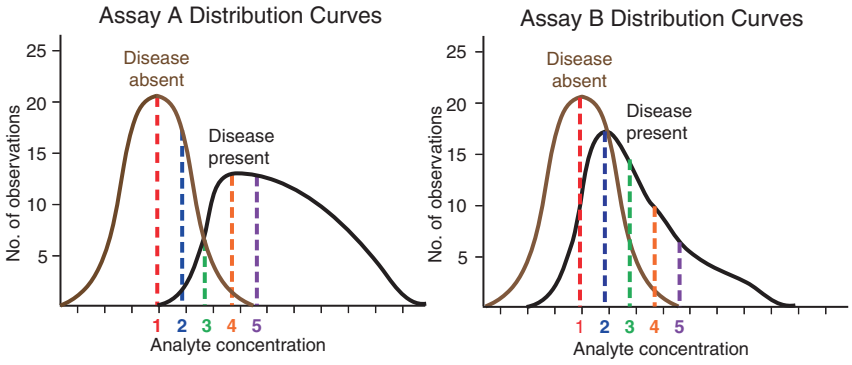
6. The ratio of disease-present to disease-absent individuals (pretest probability) is constant. Again, this criterion may be difficult to fulfill because certain disorders may be rare in a primary-care clinic but very common in a referral clinic.

C. **STA**ndards for **R**eporting of **D**iagnostic accuracy studies (STARD) are guidelines that were created to improve the completeness and transparency of diagnostic accuracy studies and reports.[58,59] A STARD checklist facilitates compliance with these guidelines and may be recommended or required by a journal's editorial board. Improved assessment and reporting leads to better diagnostic assays and improved patient care.

RECEIVER OPERATING CHARACTERISTIC (ROC) CURVES[60,61]

I. ROC curves were originally developed to assess the ability of radar images to detect enemy aircraft in World War II, and therefore to assess the ability to detect true signals from background noise. In the context of laboratory tests, the ROC curves display the relationship between a TP rate and a FP rate.
   A. *TP rate* is equal to diagnostic sensitivity expressed as a decimal (sometimes reported as a %). For example, when the diagnostic sensitivity is 90 %, the TP rate is 0.9; results are positive in 9 of 10 disease-present animals.
   B. *FP rate* is equal to 1 minus the diagnostic specificity expressed as a decimal (sometimes reported as a %). For example, when the diagnostic specificity is 70 %, the FP rate is 1 – 0.7 = 0.3, and 3 of 10 disease-absent animals would have a FP result. When the diagnostic specificity is 100 %, there would be no FP results and thus the FP rate would be 0.0.
   C. Figure 1.12 shows theoretical results from the comparison of two assays, assay A and assay B.

II. The clinical value of the comparison of diagnostic procedures by ROC curves depends on many factors. Major issues to be addressed include the following:
   A. The assays should be analytically valid and applicable to clinical investigations.
   B. Selection of the comparison groups should be clinically relevant. Both groups should have similar clinical features (e.g., both have polyuria, vomiting, or anemia) so that the ability of the assays to differentiate relevant disorders is evaluated. Conversely, comparison of a sick group versus a healthy group is probably not appropriate or needed (the animals could typically be defined as healthy without this laboratory testing).
   C. The accuracy of the comparison between diagnostic procedures is dependent on the accuracy of the differentiation between disease-present and disease-absent groups. Results of the comparison need to be interpreted accordingly.
   D. ROC curves can be used to compare the diagnostic accuracy of assays.
      1. A visual inspection of Figure 1.12 reveals that the ROC curve for assay A is closer to the top left corner than assay B, and thus assay A has better diagnostic accuracy than assay B.
      2. A more objective comparison can be accomplished by calculating the area under the curve for each assay. Generally, the greater the area under the curve, the greater the diagnostic accuracy of the assay. However, these values should be interpreted carefully if the shapes of the ROC curves differ.
   E. ROC curves can be used to help establish a decision threshold for diagnosing a disorder (Figure 1.13). This is most commonly based on the point in the ROC curve that maximizes the sum of sensitivity and specificity, i.e., the point closest to the upper left corner of the plot. Other than being used to establish decision thresholds, Figure 1.13 also provides information that should be considered when interpreting the test results of assay C.



Assay A Diagnostic Properties

Decision threshold	1	2	3	4	5
Dx sensitivity	1.00	0.98	0.90	0.80	0.60
Dx specificity	0.50	0.60	0.90	0.97	1.00
1 – Dx specificity	0.50	0.40	0.10	0.03	0.00

Assay B Diagnostic Properties

Decision threshold	1	2	3	4	5
Dx sensitivity	0.90	0.70	0.50	0.35	0.20
Dx specificity	0.52	0.70	0.90	0.97	1.00
1 – Dx specificity	0.48	0.30	0.10	0.03	0.00

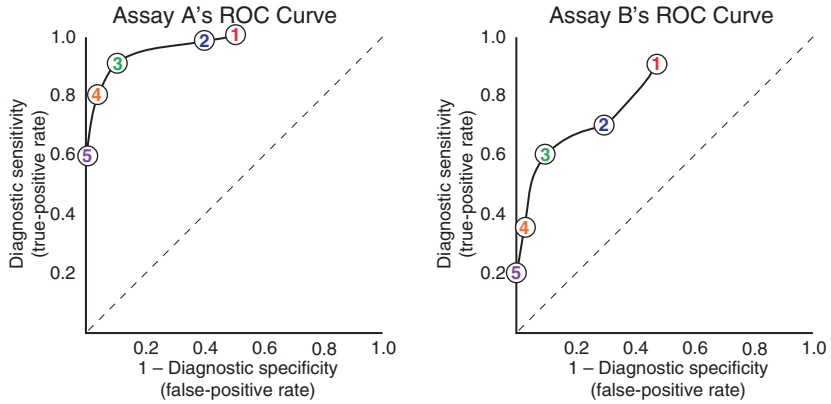


Figure 1.12. Comparison of diagnostic value of two theoretical assays by ROC curves.

- The initial step of the evaluation is the analysis of samples from two groups of animals (disease present and disease absent) by the two assays. The presence or absence of disease is established by a gold standard procedure.
- The data are plotted to obtain the distribution curves (*top curves in figure*). The assays generate different results and therefore curves for the disease group. To gather data for the ROC curves (*lower curves*), multiple decision thresholds (analyte concentrations) are selected that will provide different diagnostic sensitivity and specificity values. The decision thresholds are then used to classify actual measured concentrations as being TP, FP, TN, or FN results. For the illustration in the top graphs, five decision thresholds were selected, and the dashed lines represent the separation of positive and negative results at each decision threshold.
- From the classified data, the diagnostic sensitivity (TP rate) and specificity (TN rate) values are calculated for each decision threshold. (For this illustration, the number of animals in both groups was estimated from the graphs with an assumption that the total number in each group was equal.) The FP rate is calculated by subtracting diagnostic specificity from 1.
- The decimal fractions for TP rate and FP rate are plotted in the bottom graph. The 45° dashed line represents the ROC curve that would be obtained by random classification (e.g., flipping a coin to classify animals as disease present or disease absent). The best ROC curve approaches the top left corner of the graph where nearly all positive results are TP results, and the area under the curve is greater (not calculated here). In this comparison, assay A is a better diagnostic procedure than assay B for detecting the assessed disease.

Dx, diagnostic

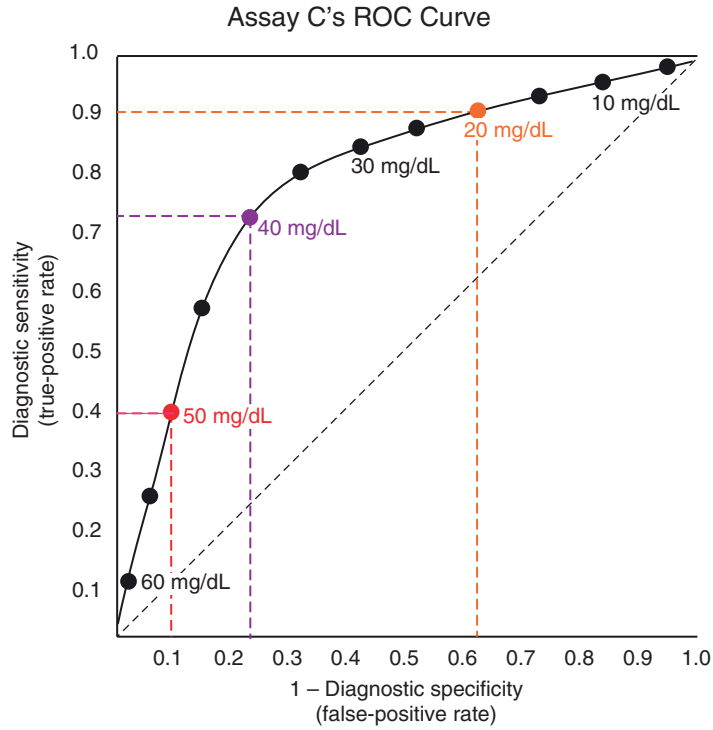


Figure 1.13. Selecting decision thresholds with ROC curves. In this theoretical assay C, the analyte concentrations are plotted on the ROC curve for assay C.

- The top-leftmost point (*purple*) provides the best balance between diagnostic sensitivity and diagnostic specificity and is often chosen as a decision threshold to conclude that a patient has a particular condition. Values of > 40 mg/dL would be considered evidence of the disease. With this decision threshold, the assay would have a diagnostic sensitivity of 73 % and a diagnostic specificity of 78 %.
- For use as a screening test, one might choose to set the decision threshold for disease at 20 mg/dL, accepting only 10 % FNs (i.e., 90 % diagnostic sensitivity). Values of > 20 mg/dL would suggest the presence of the disease. With this decision threshold, the assay would have a high diagnostic sensitivity (90 %), but a diagnostic specificity of only 37 %.
- For a confirmatory test, the decision threshold for disease may be set at 50 mg/dL to accept only 10 % FPs (i.e., 90 % diagnostic specificity, or an FP rate = 1.0 – 0.9 = 0.1). Values of > 50 mg/dL suggest that the animal has the disease. With this decision threshold, the diagnostic specificity would be high (90 %), but the diagnostic sensitivity would be only 40 %.
- One could also use the ROC curve to determine upper and lower clinical decision thresholds. A value of 20 mg/dL might be chosen as the lower decision threshold, so animals with values of < 20 mg/dL would be considered likely not to have the disease. A value of 50 mg/dL might be chosen as the upper decision threshold, so animals with values of > 50 mg/dL would be considered likely to have the disease. Based on this theoretical example, what would be the decision thresholds if you decide to accept 5 % FNs and 5 % FPs?

Answer: < 10 mg/dL – likely not to have the disease; > 55 mg/dL – likely to have the disease

1. Consider a patient with clinical signs suggestive of the disease of interest and an assay C result of 40 mg/dL.
   a. At 40 mg/dL, assay C has a FP rate near 0.22, or a diagnostic specificity of 78 %. In other words, of 100 dogs that do not have the disease of interest, about 22 will have an analyte concentration of > 40 mg/dL (22 % of those without the disease

classified as diseased). Therefore, if the result is > 40 mg/dL, the patient may or may not have the disease, but disease is more likely than not.

b. At 40 mg/dL, assay C has a TP rate near 0.73, or a diagnostic sensitivity near 73 %. So, of 100 dogs that do have the disease of interest, 73 will have an analyte concentration of > 40 mg/dL (73 % of those with the disease classified as diseased). Therefore, despite a value that is not > 40 mg/dL, the patient may still have the disease because 27 % of patients with the disease have values of 40 mg/dL or less.

c. This example emphasizes that for many diagnostic tests, animals with and without the disease of interest may have the same assay result.

2. Another concept related to this illustration is that each ROC curve represents the results obtained from one sampling of the defined populations by using one assay as it compares to a diagnosis based on one set of criteria. Another sampling using the same selection criteria, the same assay, and the same diagnostic criteria probably will not produce the same ROC curve.

F. Factors other than the probability of correctly classifying an animal as having or not having a disease can influence where decision thresholds are established:

1. If the mortality rate is very high for a disease that is not treated promptly, or if the disease is known to cause pain or unreasonable discomfort, the decision threshold may be changed so that there are fewer false negatives (greater diagnostic sensitivity).
2. If the treatment is known to have severe side effects or the financial cost of a certain diagnosis is great, the decision threshold may be changed so that there are fewer false positives (greater diagnostic specificity).

HERD-BASED TESTING FOR CATTLE

I. Other than using results of laboratory assays to help detect or establish the presence of pathologic states in individual animals, clinical laboratory assays also are used to evaluate the nutritional and metabolic status of groups of cattle. These evaluations should complement other management tools such as ration evaluation, milk composition analysis, body condition scoring, and monitoring disease incidence (retained placenta, displaced abomasum, milk fever, and ketosis). The goal of herd-based testing (metabolic profiling) is to detect subclinical laboratory abnormalities that reflect correctible nutritional management practices so that changes can be instituted prior to adverse clinical and economic consequences. Metabolic profiling may be most applicable to large herds where (i) adequate sample sizes are available, (ii) costs are diluted across more animals, and (iii) correcting a suboptimal nutritional state could translate into considerable economic savings.

II. The approach to group testing differs in several ways from individual animal testing with diagnostic profiles.[62–65]

A. Emphasis is placed on evaluating and monitoring subclinical rather than clinical disease. Clinically ill cattle are excluded from herd-based testing because their results may be indicative of an individual disease problem rather than a herd problem.

B. Individuals to be tested must belong to defined groups of similar age and lactation stage under similar environmental conditions and nutritional management (e.g., prefreshening cows and postcalving cows). Specific group trends or abnormalities may not be apparent if inclusion criteria are too broad, thus yielding more physiologic variation.

C. Useful information requires adequate group representation and therefore an adequate sample size for the group of interest. Sampling multiple animals from a defined group helps reduce variation unrelated to nutritional state.

1. Sample sizes of 7–12 have been advocated as reasonable pools to provide useful information for most purposes, regardless of herd size. However, greater numbers of test individuals provide results with tighter confidence limits and may be necessary to adequately represent target groups in large herds.
2. Herd size may limit the number of animals in a relevant group that can be tested at one time (e.g., too few prefresh cows). However, useful information may be obtained in these groups by testing over time as animals enter the group. Interpretation must then include considerations of temporal variables.
3. Economic pressures encourage smaller test groups and pooling of samples, but pooling may be undesirable.
   a. In one study, 5 of 21 analytes (including [tCa^{2+}] and [BHB]) had some pooled mean values that differed from mean values calculated from results for the individual animals.[66]
   b. Unexpected and marked abnormalities in one or more samples may bias the pooled results, especially if unequal volumes of sample from each individual are used.
   c. Individual animal data cannot be generated, so only mean values rather than ranges of values or proportions of abnormal results above or below a decision threshold can be assessed (see Section III).

D. Analytes selected for herd-based metabolic profiling tests should reflect metabolic/nutritional status. Typically, these analytes are not tightly regulated by hormonal or other physiologic factors. For example, metabolic pathways attempt to maintain [glucose] within a certain range even if there is a marked difference in the intake of carbohydrates; thus, serum [glucose] is not a good analyte for assessing carbohydrate intake or digestibility. In contrast, plasma [BHB] is not tightly regulated, and increased concentrations reflect a deficient energy status. Even if analytes meet this criterion, it is also important that analytical variation is small compared to changes caused by altered nutritional states. Examples of tests and results that may be useful include the following:
   1. Low blood hematocrit values in the first 8 wk of lactation suggest the need to evaluate energy and protein nutrition.
   2. Serum [urea] at any lactation stage is an assessment of ruminal balance of energy and available protein.
   3. High serum [BHB] at about 5–50 d into lactation is associated with suboptimal energy and glucose balance and, clinically, reduced milk production, increased clinical ketosis, displaced abomasums, and reduced fertility. Samples should be collected at a consistent time relative to feeding, e.g., 4–5 h after the first meal of the day, when rumen butyric acid production is high.
   4. High serum or plasma nonesterified fatty acid (NEFA) concentrations in prefresh dry cows in the last month of gestation (especially 2–14 d prior to calving) are evidence of negative energy balance. Samples should be collected shortly before feeding time. One can draw and freeze multiple samples and send those that fulfill the 2- to 14-d precalving criterion once this is known.
   5. Low serum [tCa^{2+}] 12–24 h after calving by multiparous cows indicates clinical or subclinical parturient hypocalcemia.

III. Interpretation

A. Abnormalities in herd-based test results must be interpreted with attention to all the variables that can affect them, not just nutritional factors. Other factors include preanalytical sample variation, analytical variation, biological variation, circadian/prandial

variation, seasonal variation, variation in physiologic states, occurrence of pathological states, and other environmental and management factors. Most of these variations are controlled by careful group selection criteria and appropriate procedures for sample collection, processing, and analysis. Preanalytical factors are detailed in specific chapters where analytes are discussed.

B. Most herd-based test results fall within diagnostic RIs because the sampled individuals lack clinical signs of disease. Therefore, a different set of interpretive guidelines is necessary to detect subclinical abnormalities. Interpretive guidelines are suggested recommendations by experts in the field based on experience and research, but it is not clear how widely applicable the guidelines are given interlaboratory variability in assay results.

C. Data have been evaluated in several ways.
   1. Mean herd values may be compared to mean values of reference herds. However, these data are not readily available because of the expense of testing and difficulties identifying suitable reference herds.
   2. Mean herd values may be compared to an established expected mean result for the analyte and group of interest. For example, it has been suggested that mean urine pH should be 6.0–7.0 in prefresh cows fed anions to help prevent milk fever. A mean pH of 6.0–7.0 supports appropriate acidification of the group. When the herd's mean ± an uncertainty interval does not include 6.0–7.0, a herd's urine pH is considered inappropriate. When the herd's mean value is not 6.0–7.0, but the mean ± uncertainty interval overlaps this target, the results are considered borderline. A 75 % confidence interval has been suggested as a useful guide for metabolic profiling and a reasonable compromise between 95 % confidence and practicality.
   3. The percentage of individual results above or below an established decision threshold can be calculated and compared to what is considered an acceptable percentage of high or low values. A percentage (± uncertainty interval) greater than the accepted percentage signals a problem. A percentage and confidence interval overlapping with the accepted percentage is a borderline result. Decision thresholds may be somewhat artificial in that the risk for some conditions increases continuously with the magnitude of the analyte (e.g., concentration of nonesterified fatty acids and displaced abomasum).
   4. Others have suggested using process control charts to graphically monitor changes of an analyte in a particular group over time. A running graph of mean values and high-low ranges of individual values can be plotted over time to evaluate for group changes that signal problems. Fluctuations must be assessed relative to established limits to determine when action should be taken.

D. Just as with population-based RIs, herd-based profiling has limitations, but useful information can be acquired for making management decisions.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 2

LEUKOCYTES

Michael A. Scott and Steven L. Stockham

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Table 2.1. Abbreviations and symbols in this chapter	
[x]	x concentration (x = analyte)
BLV	Bovine leukemia virus
C5a	Complement factor 5a
CBC	Complete blood count
CD	Cluster of differentiation or designation
CLP	Circulating lymphocyte pool
CNP	Circulating neutrophil pool
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
EDTA	Ethylenediaminetetraacetic acid
FeLV	Feline leukemia virus
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
IgE	Immunoglobulin E
IL-x	Interleukins (x for Arabic numbers)
IMN	Immune-mediated neutropenia
LAD	Leukocyte adhesion deficiency
LRL	Lower reference limit
LTB_4	Leukotriene B_4
MALT	Mucosa-associated lymphoid tissue
MLP	Marginated lymphocyte pool
MNP	Marginated neutrophil pool
MPP	Multipotent progenitor cell
MPS	Mucopolysaccharidosis
NK	Natural killer
NMP	Neutrophil maturation pool
NPP	Neutrophil proliferation pool
NSP	Neutrophil storage pool
nRBC	Nucleated erythrocyte (nucleated red blood cell)
PAF	Platelet-activating factor
PCR	Polymerase chain reaction
RI	Reference interval
TNF	Tumor necrosis factor (may be specified as α or β form)
TSLP	Thymic stromal lymphopoietin
URL	Upper reference limit
WBC	White blood cell (leukocyte)
WRI	Within reference interval

PHYSIOLOGIC PROCESSES

I. *Leukon* (from Greek *leukos*, white): all leukocytes (white blood cells, WBCs) in an animal, including WBC precursors, WBCs in blood and lymph vessels, and extravascular WBCs in all other tissues

A. Bone marrow contains precursors for neutrophils, eosinophils, basophils, monocytes (and the macrophages and osteoclasts some of them become), lymphocytes, dendritic cells, and mast cells (the last two being considered tissue WBCs).
   1. Leukopoiesis (*-poiesis* from Greek, to make) is part of hematopoiesis (hemopoiesis), which is a complex system involving stem cells that are capable of self-renewal and differentiation toward a committed cell line (Figure 2.1).
   2. Stem cells are present in blood and other tissues, including bone marrow, spleen, and liver, but they are not recognizable by routine microscopy because they look similar to small lymphocytes.
   3. Specific stimuli and regulators govern WBC differentiation and production.

B. The other major organs involving WBC production and regulation are the thymus, lymph nodes, spleen, and MALT.

C. Most WBCs in blood are in transit from sites of production to sites of function or destruction; few WBCs function within the vascular compartment.

D. Extravascular WBCs
   1. *Granulocytes* (neutrophils, eosinophils, and basophils) perform their roles in host defense and die. Granulocytes are also referred to as *polymorphonuclear* WBCs, a term that some use specifically for neutrophils.
   2. Lymphocytes may be stimulated to transform and proliferate (undergo blastogenesis), return to blood via lymphatic vessels, or die.
   3. Monocytes can transform into dendritic-like cells or macrophages that perform their host defense functions and die. Macrophages may also proliferate or develop into osteoclasts.
   4. Immature dendritic cells distribute to organs throughout the body, particularly those in contact with the outside world. When mobile dendritic cells contact a presentable antigen, they mature and migrate through blood or lymphatics to spleen or lymph nodes, present antigen to T lymphocytes, and eventually die.
   5. Mast cell precursors differentiate into tissue mast cells (mostly outside the bone marrow), perform their roles in host defense, and eventually die. (Although sometimes classified as granulocytes in human medicine, they have not been considered granulocytes in veterinary medicine.)

II. Major WBC functions

A. Together, WBCs help maintain homeostasis through surveillance and innate and adaptive responses to external (e.g., microorganisms) and internal (e.g., atypical cells) threats, and by promoting repair of damaged tissue and removal of dead cells. These activities are ongoing, involving the generation of billions of WBCs each day, but they are clinically silent until a threat is too great or responses go awry.

B. With clinical or subclinical disease, routine laboratory testing may reveal WBC abnormalities that reflect pathologic or physiologic states. Interpretation of their significance is aided by an understanding of the major functions of WBCs seen in blood (Table 2.2). However, the roles of some of these cell types are far from clear, new subtypes of WBCs (e.g., neutrophils, monocytes, basophils) are being identified, and species differences are largely unknown.

III. Neutrophil pools and kinetics (movement of neutrophils) (Figure 2.2)

A. Marrow neutrophils
   1. G-CSF, GM-CSF, IL-1, IL-3, IL-6, and some products of microorganisms all stimulate granulopoiesis. G-CSF, the major regulator of neutrophil production, promotes

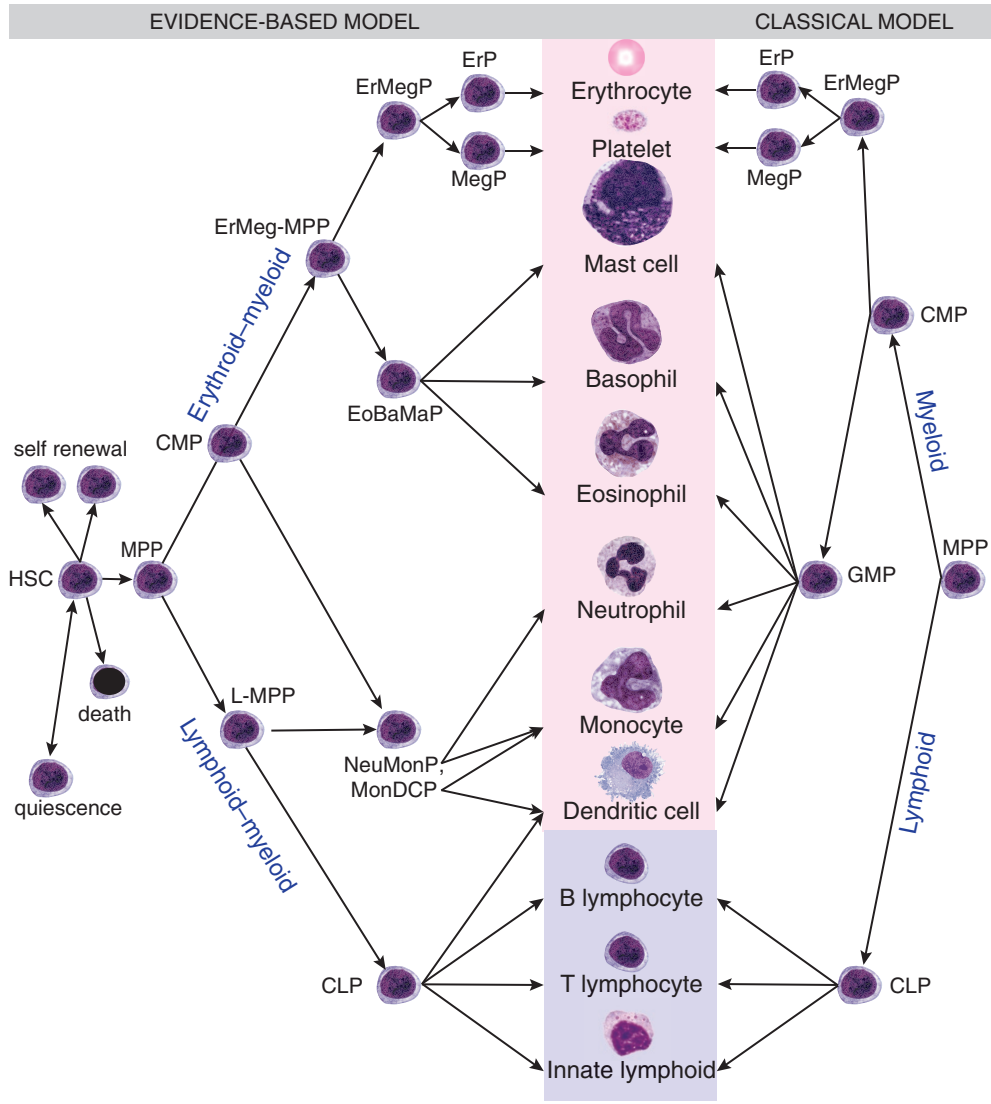


Figure 2.1. Schematic models of hematopoiesis. Each day, billions of blood cells are formed from a renewing pool of hematopoietic stem cells (HSCs) that can become activated and differentiate into multipotent progenitor (MPP) cells. In general, MPPs respond to environment signals by dividing and progressing through a series of increasingly lineage-restricted progenitor cells to committed hematopoietic precursors that undergo terminal differentiation and maturation to RBCs, platelets, and WBCs.

- Classical model (right side): Contrary to current evidence (e.g., leukemic cells expressing both lymphoid and myeloid markers), the classical model of hematopoiesis was based on initial differentiation of MPP cells into a common myeloid progenitor (CMP) and a distinctly separate common lymphoid progenitor (CLP). In this model, the CLP forms all the lymphocytes and the CMP generates (i) a granulocyte/monocyte progenitor (GMP) leading to nonlymphoid WBCs and (ii) an erythroid/megakaryocyte progenitor (ErMegP) leading to RBCs and platelets through an erythroid progenitor (ErP) and a megakaryocyte progenitor (MegP), respectively.

commitment of progenitor cells to the myeloid lineage (CFU-G), promotes proliferation of precursors in the proliferative neutrophil pool (NPP, mitotic pool), accelerates maturation of cells in the maturation pool (NMP, postmitotic), and promotes release of neutrophils to blood.

2. Myeloblasts, progranulocytes (promyelocytes), and myelocytes divide and mature in the NPP. In healthy mammals, neutrophil precursors are in this pool for about 3 d.
3. In the NMP, metamyelocytes mature to band neutrophils and then to segmented neutrophils. Segmented neutrophils that are ready for release to marrow sinusoids are in a subpool called the NSP (neutrophil storage pool). In health, neutrophilic cells are in the NMP for about 4 d in dogs.[4] The NMP:NPP ratio is about 4–6, meaning that the number of cells in the NMP is 4–6× the number of cells in the NPP.
4. In health, segmented neutrophils are retained in the marrow largely by sinusoidal endothelium, adhesion factors, and a balance of chemokines.[5] G-CSF, a major factor that promotes migration of neutrophils from marrow to blood, alters this balance and reduces neutrophil retention in the marrow.
    a. G-CSF effect on chemokines: The chemokine CXCL12 (stromal-derived factor 1, SDF1) is constitutively released from stromal cells, including osteoblasts, and promotes neutrophil retention in the marrow by binding to its receptor (CXCR4) on neutrophils. In contrast, CXCL2 expressed on endothelial cells (and megakaryocytes) promotes egress to marrow sinusoids via binding to the neutrophil receptor CXCR2. G-CSF rapidly decreases CXCL12 production, promotes

Figure 2.1. *continued*

- An evidence-based model: Evidence from studies of mice and people supports incomplete separation of lymphoid and myeloid lineages, with the MPP instead diverging down more flexible lymphoid–myeloid and erythroid–myeloid paths (*left side*).
    - Lymphoid–myeloid path: This path begins with formation of a so-called lymphoid-primed MPP (L-MPP) that can ultimately generate (i) neutrophils and monocytes via a neutrophil/monocyte progenitor (NeuMonP) and (ii) all lymphocytes and at least plasmacytoid dendritic cells, either directly or through a CLP as shown.
    - Erythroid–myeloid path: The MPP forms an erythroid/megakaryocyte MPP (ErMeg-MPP) that likely develops into a progenitor for eosinophils, basophils, and mast cells (EoBaMaP) as well as the ErMegP as in the classical model. There may be a CMP prior to the ErMeg-MPP that can lead to neutrophil, monocyte, and dendritic cell production, as shown. A monocyte/dendritic cell progenitor (MonDCP), whether from the erythroid–myeloid path or the lymphoid–myeloid path, gives rise to monocytes and all subtypes of dendritic cells.

Other steps and variations may occur as well, and some steps may be bypassed, such as the MPP leading directly to an ErMegP or a MegP. In addition, there may be species differences, but the concept of mixed lymphoid and myeloid progenitors fits with mixed phenotypes of some human and veterinary neoplasms.[1–3]

CLP, common lymphoid progenitor; CMP, a common myeloid progenitor; EoBaMaP, eosinophil/basophil/mast cell progenitor; ErMegP, erythroid/megakaryocyte progenitor; ErP, erythroid progenitor; GMP, granulocyte/monocyte progenitor; HSC, hematopoietic stem cells; L-MPP, lymphoid-primed MPP; MPP, multipotent progenitor; NeuMonP, neutrophil/monocyte progenitor

Table 2.2. Major homeostatic functions of the WBC types seen in blood

Cell type	Major functions
Neutrophil	Phagocytosis of invading microbes (major bacterial phagocyte) Microbial killing via myeloperoxidase-mediated oxidative injury, enzymes, antimicrobial peptides, and neutrophil extracellular traps (NETs) Immunoregulation via cytokines and surface molecules
Eosinophil	Damaging helminths, bacteria, and viruses by release of granule contents (eosinophil peroxidase, major basic protein, and the ribonucleases eosinophil cationic protein and eosinophil-derived neurotoxin) or formation of antibacterial eosinophil extracellular traps Immunoregulation: regulation of thymic T-cell development, recruitment of type 2 T helper lymphocytes, antigen presentation to T lymphocytes, promotion of humoral immunity, activation of dendritic cells, recruitment of macrophages, activation of neutrophils and mast cells Promotion of tissue repair via release of growth factors Phagocytosis (minor role)
Basophil	Protection against ecto- (e.g., ticks) and endoparasites (e.g., helminths) Participation in immediate and late-stage immune reactions through release of a variety of substances (e.g., histamine, heparin, leukotriene, PAF, antimicrobial peptides) in response to innate or adaptive immune stimuli (e.g., IgE, C5a, bacterial products, cytokines) Immunoregulation via release of cytokines (e.g., IL-4)
Monocyte	Phagocytosis of opsonized or abnormal cells and material (certain microorganisms, foreign material, dead cells, and neoplastic cells) Antigen presentation to T lymphocytes Immunoregulation via release of numerous cytokines Differentiation to macrophages and dendritic cells
B lymphocyte[a]	Recognition of nonself antigens Production of antibodies to eliminate pathogens or pathogen-infected cells Differentiation to plasma cells Generation of memory cells to respond to specific antigens in the future Immunoregulation via release of cytokines
T lymphocyte[a]	Recognition of nonself antigens Nonphagocytic killing of antigen-bearing abnormal cells (infected, neoplastic) via contact-dependent release of granule contents Generation of memory cells to respond to specific antigens in the future Immunoregulation via release of cytokines
NK cell[a]	Innate recognition of abnormal cells (infected, neoplastic) via altered cell membrane protein expression (MHC-I and related molecules) Antibody-independent and Fc-mediated antibody-dependent cytotoxicity via release of granule contents Immunoregulation via release of cytokines

[a] These 3 lymphocyte subtypes cannot be specifically identified by routine light microscopy, but lymphocytes with cytoplasmic granules are T lymphocytes or, less often, NK cells.

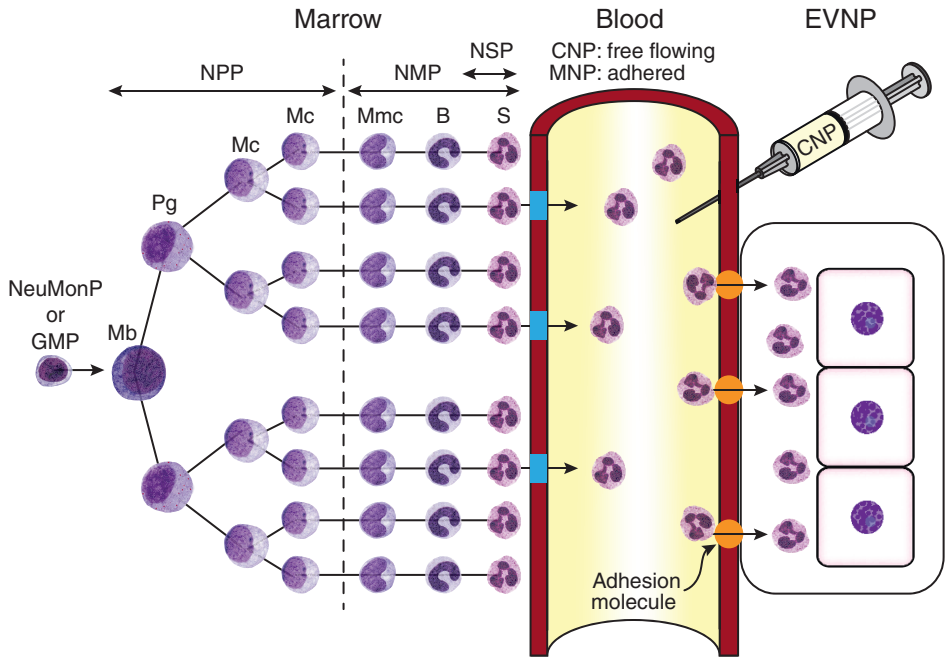


Figure 2.2. Neutrophil kinetics in health. Marrow has three major neutrophil pools: (i) self-renewing neutrophil/monocyte progenitors (NeuMonP) or granulocyte/monocyte progenitors (GMPs), (ii) a NPP (mitotic pool) that contains myeloblasts (Mb), progranulocytes (Pg, also called promyelocytes), and myelocytes (Mc), and (iii) a NMP (postmitotic maturation pool) that contains metamyelocytes (Mmc), band neutrophils (B), and segmented neutrophils (S). A NSP within the NMP contains segmented neutrophils. When neutrophils leave the marrow and enter blood, they distribute between the MNP and the CNP. After neutrophils bind to adhesion proteins on endothelial cells, they migrate into tissues to form the EVNP (extravascular neutrophil pool). In the EVNP, neutrophils perform their protective functions and die.

CXCR4 cleavage from neutrophils, and increases endothelial CXCL2 expression, thus promoting their transcellular migration across the sinusoidal endothelium and into systemic blood.

b. G-CSF effect on adhesion molecules: The integrin VLA-4 (very late antigen-4, CD49d/CD29) expressed on bone marrow neutrophils also promotes neutrophil retention in bone marrow by binding the cells to stromal VCAM-1 (vascular cell adhesion molecule 1, CD106). G-CSF decreases VCAM-1 expression.

c. Other neutrophil-releasing factors include chemoattractants (C5a, IL-8, n-formyl-methionyl-leucyl-phenylalanine, LTB_4, and PAF) and cytokine leukocytosis factors (IL-1, IL-6, TNFα, TNFβ, and GM-CSF).

B. Blood neutrophils

1. In health, neutrophils are thought to have a blood half-life of about 5–10 h. However, their overall half-life is controversial and may be as long as 3–4 d, depending on how long they can survive in tissue and how much they might exit blood and return to it by reverse migration.[6]
2. Blood neutrophils are distributed into two pools as determined by their location in vessels.

   a. Neutrophils that are free flowing in blood (and thus collected in blood samples) are in a CNP.
   b. Neutrophils that temporarily adhere to endothelial cells are in the MNP, which is located primarily in small capillaries, postcapillary venules, or sinusoids where blood flow is slow and neutrophils have the most opportunity to contact endothelial cells (potentially in lungs, liver, spleen, and bone marrow). After adhesion, neutrophils may break loose and reenter the CNP (e.g., with increased blood flow from activity or epinephrine) or migrate into the extravascular neutrophil pool (EVNP).
   c. In most mammals, the MNP:CNP ratio is near 1. In cats, the ratio is ~3.[7]
3. Major processes that influence measured blood neutrophil concentrations
   a. Production
      (1) Presence and amount of hematopoietic stimuli
      (2) Stem cell proliferation and differentiation
      (3) Effectiveness of maturation
   b. Release from hematopoietic tissue: The oldest or most mature neutrophils preferentially leave the marrow because they have decreased expression of retention factors (CXCR4 and VLA-4) and increased expression of receptors (CXCR2) for the egress-promoting chemokine CXCL2.[8,9]
   c. Distribution of neutrophils between the CNP and MNP
   d. Migration out of blood: Compared to less mature neutrophils, mature segmented neutrophils have more potential to emigrate from blood.
4. If neutrophils circulate into inflamed tissue, they may leave blood through an active process involving loose adhesion, firm adhesion, and migration, mostly through postcapillary venules.[10] This requires 5–45 min.
   a. Inflammatory cytokines (including IL-1 and TNF from macrophages and IFNγ from lymphocytes) stimulate endothelial cells to produce and express adhesion proteins (selectins) that mediate neutrophil "rolling" (on-and-off adhesive interactions) by binding to complementary ligands on neutrophils. Neutrophils also express selectins that bind to complementary ligands on endothelial cells. These interactions can overcome the forces of flowing blood and tether neutrophils to the vessel wall.
   b. Endogenous chemical mediators, including LTB_4 and PAF, activate neutrophils, leading to expression of high-affinity membrane proteins called integrins. A neutrophil integrin (CD11a/CD18) binds to endothelial cell receptors (intercellular adhesion molecules 1 and 2) resulting in tight adhesion.
   c. Tightly adhered neutrophils undergo cytoskeletal rearrangement allowing them to crawl along the luminal surface of endothelial cells and migrate through endothelial cell junctions (mostly) and through endothelial cells (less often). Neutrophils provide alternative binding partners that allow "unzipping" of tight junctions as they zip themselves through the vessel wall between cells.
   d. Outside the vessel, neutrophils crawl along pericytes, then move between them, penetrate the basement membrane, detach, and continue along a chemical gradient to a chemotactic source (e.g., LTB_4, C5a, IL-8, PAF, bacterial products).
5. Based on murine studies, it has been proposed that as neutrophils age in circulation, their expression of the chemokine receptor CXCR4 increases while that of the chemotactic receptor CXCR2 decreases, thus promoting homing and transendothelial migration back into the CXCL12-rich bone marrow where the cells undergo apoptosis and phagocytosis by resident macrophages.[5,6] Such phagocytic clearance of senescent

neutrophils in the bone marrow may promote local production of G-CSF and therefore stimulate neutropoiesis to maintain neutrophil numbers. In contrast, with removal of neutrophils by macrophages at inflammatory sites, G-CSF production appears to decrease, thus limiting inflammation. Control of neutrophil production differs between homeostatic and inflammatory states and is less understood during homeostasis.

C. Extravascular neutrophils
   1. Most neutrophils that have migrated out of the blood are thought to perform their inflammatory functions, die by apoptosis or other means, and undergo phagocytosis, never returning to blood.
   2. However, there is evidence that some neutrophils can migrate away from an inflamed site and return to blood via lymphatics or reverse transendothelial migration across blood vessels.[6,11,12]
   3. In the absence of disease, most of an animal's neutrophils are thought to die after movement into respiratory airways or the alimentary tract lumen, or they are removed in the liver, spleen, and bone marrow as discussed above for pre-apoptotic blood neutrophils.

IV. Lymphocyte pools and kinetics
   A. Primary lymphoid organs: bone marrow and thymus
      1. B lymphocytes, T lymphocytes, and innate lymphoid cells (including NK cells) are produced from hematopoietic stem cells in hematopoietic tissue, primarily the bone marrow in domestic mammals (Figure 2.1).
      2. Cells that will become B lymphocytes may mature in the bone marrow or in other tissues, likely the intestine. In birds, they mature in the bursa of Fabricius, a cloacal diverticulum, which is the origin of the "B" label.
      3. Cells that will become T cells require maturation in the thymus (origin of the "T" label). The thymus involutes in young animals after a large repertoire of T cells is created.
   B. Secondary lymphoid organs and tissues: lymph nodes, spleen, and MALT
      1. Secondary lymphoid organs contain organized lymphoid tissue with T and B lymphocyte components, including follicles. They provide for detection of antigenic substances in blood and lymph, and they are important sites in the initiation and control of adaptive immune responses.
      2. Lymph nodes are widely distributed to receive afferent lymphatic fluid from most of the body. With the fluid comes potential antigens as well as lymphocytes and dendritic cells. Lymphocytes and some dendritic cells also enter lymph nodes through an active process via high endothelial venules and specific adhesion molecule interactions.[13,14]
         a. Dendritic cells provide a means of detecting antigens throughout the body and bringing them to lymphoid tissue for processing.
         b. The lymphocytes that enter a lymph node traverse it along a chemotactic gradient, contacting many cells along the way and potentially stimulating expansion of a reactive clone. Most lymphocytes leave after a few hours to a day through efferent lymphatics that ultimately reach the thoracic duct where lymphocytes enter blood for distribution to other tissues.
      3. The spleen contains a large number of lymphocytes (white pulp), but it has no afferent lymphatics. Instead, lymphocytes enter the spleen from blood. Arterioles release blood into vascular sinuses (red pulp) near the margin of red and white pulp, and blood cells travel through the red pulp to exit in veins.[15,16] Mixing in the marginal zone between red and white pulp promotes immune cell interactions. From there, some lymphocytes detour through the white pulp; T lymphocytes are attracted to periarteriolar tissue forming

periarteriolar lymphoid sheaths, and B lymphocytes are attracted to nearby follicles. Both cell types then migrate to the red pulp, likely involving a chemical gradient, and leave the spleen in veins. A typical lymphocyte spends several hours traversing the spleen. Efferent lymphatic drainage may play a small role in lymphocyte transit.

4. MALT is nonencapsulated secondary lymphoid tissue important in surveillance of external boundaries: intestine (GALT, G for gut), airways (BALT, B for bronchial), nasal sinus (NALT), conjunctiva (CALT), tonsils, and the genitourinary tract. MALT lymphocytes routinely travel along lymphatics to draining lymph nodes and then to blood and back to MALT or other sites where they perform their functions. Blood lymphocytes enter MALT through high-endothelial venules as in lymph nodes, but via different combinations of adhesion molecules that allow for directed trafficking to particular tissues.

C. Blood lymphocytes
   1. In health, constant recirculation from blood to lymphoid or other tissue and back to blood results in the complete turnover of the blood lymphocyte population many times each day. A lymphocyte typically remains in blood for less than an hour.
   2. Like neutrophils, lymphocytes are distributed into two pools, the CLP and the MLP; shifting between pools likely occurs with changes in blood flow and changes in expression of cell adhesion molecules.
   3. T lymphocytes predominate in the blood of domestic mammals (about 60–80 % of lymphocytes), but most lymphocyte types cannot be differentiated by using routine microscopy. Lymphocytes containing cytoplasmic granules are T lymphocytes or NK lymphocytes.

D. Major processes that influence measured blood lymphocyte concentrations
   1. Production
      a. Stem cell proliferation and differentiation (mostly bone marrow)
      b. Post-activation proliferation (other tissue)
   2. Distribution of lymphocytes between the CLP and the MLP
   3. Extravasation to secondary lymphoid organs or other tissues
   4. Migration from lymph nodes via efferent lymphatic vessels to blood
   5. Loss from the body or lymphocyte death

E. Lymphocyte life span varies from hours to years, with most lymphocytes probably living weeks to months.[17,18] Naïve lymphocytes (not yet activated by a specific antigen) can live years, whereas memory and effector lymphocytes do not; the persistence of immunologic memory is greater than the life span of memory lymphocytes determined to date. Species differences in life spans are expected.

F. Lymphocytes in nonlymphoid tissues
   1. Some lymphocytes, especially memory cells, emigrate to nonlymphoid tissues such as skin through flat- or high-endothelial venules in a process mediated by specific sets of adhesion molecules and chemokines.[13] These cells can return to lymph nodes via lymphatics.
   2. Lymphocytes that become activated in tissue may be retained there or return to secondary lymphoid organs via lymphatics. There, they may recruit other cells and undergo reactive transformation and proliferation to yield cells that circulate through blood to specific targeted sites.
   3. Tertiary lymphoid tissue arises in response to inflammation (e.g., with infections, autoimmune disease, or cancer) and may resemble secondary lymphoid tissue in organization and function.

V. Monocyte pools and kinetics
   A. Monocytes and neutrophils share a common progenitor cell that is stimulated to differentiate by cytokines, particularly macrophage colony-stimulating factor (M-CSF, CSF-1) and GM-CSF (CSF-2) (Figure 2.1).
   B. Monocytes develop from monoblasts and promonocytes and typically account for < 2 % of nucleated cells in the bone marrow during health. In people, post-mitotic maturation requires 1–2 d but is shortened with inflammation. Monocytes emigrate from the marrow to blood vessels through a process involving monocyte chemoattractant protein 1 and its monocyte receptor (CCR2).
   C. When released from marrow, monocytes distribute between marginated and circulating pools. The monocyte marginated pool differs from the neutrophil marginated pool in that it includes a subset of monocytes that generally remain intravascular and marginated to fulfill their major function of patrolling vascular endothelium.[19] It also includes a relatively large reservoir of monocytes that are retained in the bone marrow blood vessels and splenic red pulp by chemokine interactions; these cells can be mobilized within minutes.
   D. Subtypes of monocytes with different functions and immunophenotypic markers have been characterized in mice and people and are recognized in some domestic mammals, including dogs, but they are not distinguished by routine microscopy.[20–22]
      1. Classical (inflammatory) monocytes are the predominant subtype (> 80 %) in circulating blood. In people, ~99 % remain in blood for ~1 d before leaving the circulation by migration or death. Some classical monocytes may be marginated in low-flow vessels, and they are a substantial component of the intravascular bone marrow marginated pool.
      2. About 1 % of the classical monocytes appear to remain in the vasculature and differentiate into intermediate and then nonclassical (patrolling) monocytes with life spans of about 4 and 7 days, respectively (in people). These monocytes are largely marginated, crawling along the endothelium; they account for < 20 % of the circulating monocytes in health but may demarginate with changes in blood flow induced by exercise or catecholamines.
   E. Like other WBCs, monocytes that emigrate to tissues do so via adhesion molecules and chemokines that promote binding to endothelial cells and movement through them. Once in tissues, monocytes may differentiate into cells of the mononuclear phagocyte system, a body-wide network of cells with phagocytic and antigen-presenting roles.
   F. The mononuclear phagocyte system includes macrophages, dendritic cells, and monocytes, populations that overlap in appearance and function and that have not been classified and named consistently. For example, the cutaneous Langerhans cell has long been considered a dendritic cell because of its dendritic appearance and migration to lymph nodes for antigen presentation, but it has also been considered a macrophage because of its embryonal origin and self-renewal capabilities.[23,24]
      1. Most macrophages do not develop from adult bone marrow monocytes but are instead derived from embryonic or fetal hematopoietic cells and replenished by self-renewal. This applies, for example, to Kupffer cells, alveolar macrophages, splenic red pulp macrophages, and microglial cells. Some resident tissue macrophages also develop from bone marrow monocytes; this is particularly important for intestinal and cutaneous tissue. Cells with a typical appearance of macrophages are not expected in blood of healthy mammals.
      2. Dendritic cells, including conventional, plasmacytoid, and monocyte-derived subtypes, are mobile or, less often, fixed cells whose major role is antigen presentation, thus linking the innate immune system to adaptive immunity. They are derived from a monocyte-dendritic cell progenitor (or a B-cell-biased lymphoid progenitor from some plasmacytoid

dendritic cells), but dendritic cell differentiation is not detectable through microscopic evaluation of bone marrow. Similarly, they are rare and not detectable in blood smears. They acquire their dendritic appearance after leaving the blood.

3. As noted, some monocytes remain in the vasculature and others migrate to tissues where they perform their functions or are thought to differentiate into macrophages and either dendritic or dendritic-like cells. Inflammation alters populations of macrophages and dendritic-like cells in a tissue, likely because of an influx of marrow-derived cells; however, resident cells may also contribute via mitosis.

G. Major processes that influence blood monocyte concentrations are production, release from hematopoietic tissue, distribution between circulating and marginated pools, and migration out of blood.

VI. Eosinophil pools and kinetics

A. Eosinopoiesis is promoted nonspecifically by cytokines, including GM-CSF, and specifically by IL-5, which is produced by several cell types, including T lymphocytes, NK lymphocytes, mast cells, some epithelial cells, and eosinophils. IL-5 promotes eosinophil development and release.

B. Eosinophils that leave the bone marrow distribute to marginated and circulating pools. Their life span in circulation is thought to be just over 1 d in people (about 2.5× that of neutrophils).[25] They emigrate to tissue by the multistep process of WBC rolling, firm adhesion, and transendothelial migration, all controlled by adhesion molecules and chemokines including eotaxins.[26]

C. Eosinophils are largely extravascular cells. In health, they are present in many tissues, but particularly the intestinal lamina propria where they are important for IgA-mediated immunity. They can live several days to weeks outside the blood.

D. Blood eosinophil concentrations are affected by production, release from hematopoietic tissue, distribution between circulating and marginated pools, and migration out of blood.

VII. Basophil pools and kinetics

A. Basophils originate in bone marrow (Figure 2.1), where their production and differentiation are controlled predominantly by IL-3 (mostly from T lymphocytes) and TSLP from stromal cells and some epithelial cells.[27] IL-3 promotes production, maturation, and function of basophils, but it also promotes mast cell production. TSLP is more specific for basophils. Circulating products of microorganisms also appear to contribute to regulation of basophil production.

B. Two functionally but not microscopically distinct subtypes of basophils have been recognized, one produced in response to IL-3 that undergoes IgE-mediated activation and one elicited by TSLP that undergoes IgE-independent activation.

C. The marrow transit time of human basophils was estimated to be ~4 d, their circulating life span is ~3–4 d, and they may survive for as long as 2 wk once they emigrate from blood.

D. Basophil emigration from blood is promoted by IL-1, TNFα, and endotoxin and appears to be similar to the process used by other WBCs. Basophils are present in low concentration in the blood of domestic mammals.

VIII. Mast cell kinetics[28,29]

A. Mast cell precursors are present in the bone marrow (Figure 2.1) and are ultimately derived from a committed stem cell that is different from the basophil's committed stem cell. Production is supported by IL-3 and stem cell factor, the latter via binding to its tyrosine kinase receptor c-kit.

B. Undifferentiated but committed mast cells leave marrow in very low numbers and circulate in blood but are recognized only by special methods that are not used clinically. They constitutively home to tissues via selective adhesion molecules and chemokine interactions, particularly to mucosal surfaces (e.g., intestine, lung) and perivascular tissue.
C. Once extravasated, mast cell precursors may function without differentiation, proliferate via mitosis, differentiate into mast cells, or die.
   1. Mast cell precursors differentiate into structurally, functionally, and chemically different mast cell subtypes based on the microenvironment. Mature mast cells are generally considered terminal cells that do not divide.
   2. Mast cells can regranulate after degranulation and have been documented to live weeks and months; they may live for years.
D. Finding differentiated mast cells in the blood of domestic mammals reflects their entry from tissues in which they matured; it is considered a pathologic state.

ANALYTICAL PRINCIPLES AND METHODS

I. *Leukogram* (leukocyte picture): portion of a complete blood count (CBC, see Appendix A) that includes results of assays and calculated data characterizing the WBCs in blood
   A. Morphologic (microscopic) evaluation
      1. A microscopic evaluation of stained WBCs is an important part of the leukogram, especially when there are increases or decreases in WBC concentrations. It helps confirm or refute automated quantitative results and provides additional diagnostic and prognostic information that cannot be obtained without microscopy.
      2. Later sections of this chapter describe the clinical significance of WBCs with abnormal nuclei, cytoplasm, sizes, inclusions, or other features associated with pathologic states. In vitro changes caused by poor sample handling can make accurate identification and characterization of WBCs difficult or impossible.
   B. [WBC]
      1. *[WBC]* is the number of WBCs per unit volume of blood (in clinical jargon, commonly referred to as *WBC count*, but WBCs are counted to calculate concentrations). The [WBC] by itself is of limited value without assessing the concentrations of each type of WBC.
      2. By most methods, nRBCs are included in the measurement for [WBC] because they are nucleated cells. Some laboratories report a total nucleated cell concentration (including nRBCs and WBCs), but it is more common for a [WBC] to be reported after correcting for the presence of nRBCs (see Section II.D).
      3. When assessed by analyzers, the [WBC] may be falsely increased by marked lipemia or platelet clumping.[30] In samples assessed by ADVIA analyzers, many Heinz bodies might cause marked false increases in the [WBC] measured in the basophil channel.[31,32] By any method, the [WBC] may be falsely decreased by sample clotting and perhaps by cell lysis in old samples.
      4. Unit conversion: /μL × 0.001 = $\times 10^9$/L (international system of units)
   C. Differential WBC count
      1. A *differential WBC count* is a method of determining the relative numbers (percentages) of the WBCs in blood.
      2. Raw data may be reported as percentages (e.g., 80 % neutrophils). These percentages are used with the [WBC] to calculate the concentrations of each type of WBC in a blood sample. It is the concentrations that should be interpreted, not the percentages.

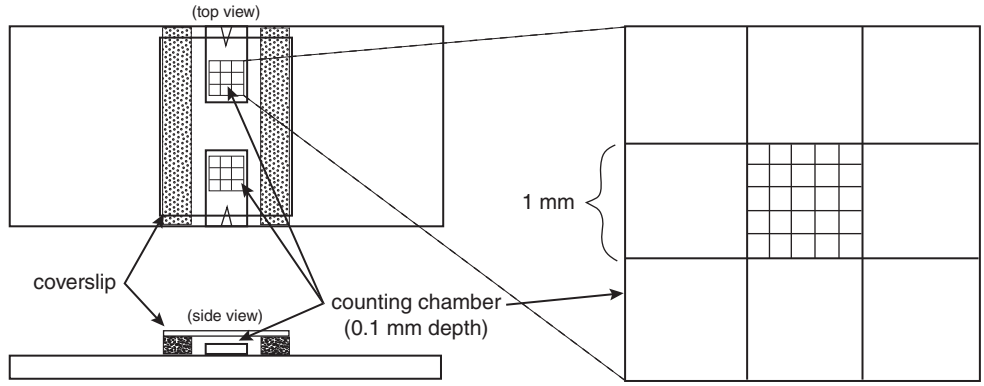


Figure 2.3. Neubauer hemocytometer. The hemocytometer has two components: (i) the thicker glass with special recessed, etched grids on each side and (ii) a glass coverslip. Each grid has nine 1 mm^2 squares used for WBC counting, and each central square is divided into 25 smaller squares that are used for RBC and platelet counting. The distance between the grid surface and the coverslip is 0.1 mm; thus, the volume of the space above the grid is 0.9 mm^3 (0.9 μL). See Figure 21.2 for more details, but briefly, after loading both chambers with well-mixed, diluted, anticoagulated blood, WBCs are counted in all nine large squares of each grid (or fewer squares with greater cell concentrations). If the two sides do not yield adequately consistent results (e.g., within 20 % of each other), the sample is replated. The [WBC] is calculated based on the 0.1 μL volume under each large square (1 mm × 1 mm × 0.1 mm depth), the sample dilution factor, and the number of large squares assessed (Eq. 2.1).

II. Principles of determining WBC concentrations
   A. Hemocytometer method
      1. A hemocytometer (Figures 2.3 and 21.2) is used to determine [WBC] by diluting the blood (usually with a diluting reservoir and capillary tube assembly) and then dispensing the diluted blood into the hemocytometer chambers formed beneath a cover glass. The WBC diluent contains a lysing agent that ruptures the RBCs, so they do not interfere with the nucleated cell enumeration. The [WBC] is calculated based on the dilution factor and number of WBCs seen in the chambers (Eq. 2.1).

$$[\text{WBC}]\ (/\mu\text{L}) = \frac{\#\ \text{cells}}{\text{volume assessed}\ (\mu\text{L})} \times \text{dilution factor}$$

$$\text{Volume assessed}\ (\mu\text{L}) = \#\ \text{large squares assessed} \times 0.1\ \mu\text{L}$$

$$\text{so: } [\text{WBC}]\ (/\mu\text{L}) = \frac{\#\ \text{cells}}{\#\ \text{large squares assessed} \times 0.1\ \mu\text{L}} \times \text{dilution factor}$$

with a blood sample diluted 1:100 and 180 cells counted in all 18 large squares (9 per side):

$$[\text{WBC}] = \frac{180}{18 \times 0.1\ \mu\text{L}} \times 100 = 10{,}000/\mu\text{L} \qquad (\mathbf{2.1})$$

      2. A microscopic differential WBC count is used in combination with the hemocytometer [WBC] to calculate concentrations of each WBC.
   B. Automated methods: Hematology analyzers provide a [WBC] (or total nucleated cell concentration if nRBCs are included) and percentages and concentrations of specific WBC types or groups of them. In contrast to the microscopic differential WBC count,

automated analyzers differentiate thousands of WBCs, so sampling error is typically not a problem. However, the electronic methods must be species specific and may not be accurate when atypical cells (e.g., toxic neutrophils, reactive lymphocytes) are present, in which case microscopic evaluation is particularly important. Automated differential counts may be considered reliable when cell populations are clearly separated as indicated by defined criteria. Several automated methods have been used, often in combination.

1. Electrical impedance method (Figure 2.4)
    a. Within the analyzer, an RBC lysis agent is added to a blood sample, the sample is diluted in a conductive electrolyte solution, a low-voltage direct current is passed through the sample, and cells are drawn through an aperture between two electrodes. When a cell (or other very poorly conductive particle) passes through the aperture, it creates electrical interference measured as a voltage pulse.

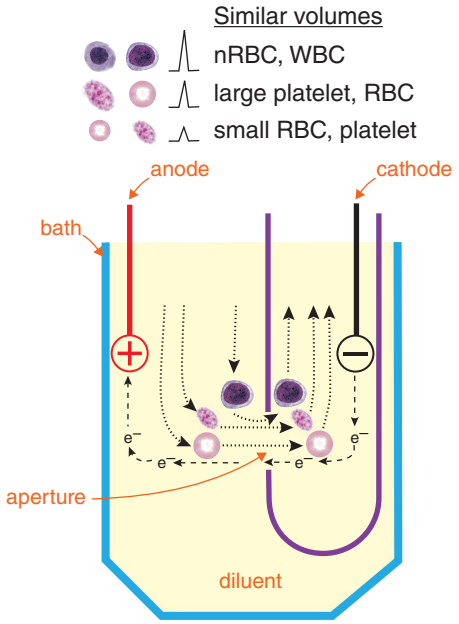


Figure 2.4. [WBC] via impedance method. Blood cells suspended in an isotonic diluent enter a bath, pass through a small aperture (about 100 µm diameter), and then leave the bath (flow shown by *dotted arrows*). Concurrently, electrons (e^-) are moving from a cathode, through the aperture, to an anode (flow shown by *dashed arrows*). When a cell passes through the aperture, it displaces electrons and interrupts the current briefly, thus creating a voltage peak than can be viewed via an oscilloscope. Each voltage peak represents a cell (a particle) passing through the aperture; the height of the peak corresponds primarily to the volume of the cell. Particles within a certain impedance range are considered RBCs, particles in a lower range are considered platelets, and other particles are not recognized as cells (if the instrument has a "voting out" program). WBCs are counted after addition of lysing agent to remove RBCs and platelets. Falsely increased [WBC] may occur with nRBCs and rarely with clumps of cryoglobulins (fibrinogen or immunoglobulin) or large lipid globules from lipid contamination or certain types of lipemia. Note for table above figure: When used to evaluate RBCs, large platelets (macrothrombocytes) may be identified as RBCs and small RBCs (microcytes) may be identified as platelets.

   b. The number of pulses detected within a defined volume of diluted blood represents a cell (particle) concentration. The degree of interference depends on the apparent volume of the particle (which can be influenced by shape and orientation within the aperture), and apparent volumes are used to differentiate particles. The volume of each particle is recorded so that an average volume can be calculated.
   c. WBC types overlap in size, so impedance alone cannot differentiate all WBCs. Differentiation may be further impaired by abnormal cell populations or abnormally large cell populations.
   d. WBCs may be mimicked by other particles, most often nRBCs, but also unlysed RBCs, giant platelets, platelet clumps, and rarely cryoproteins, large lipid particles, and certain organisms, singly or in clumps. Lyse-resistant RBCs may develop in certain disorders, and there are species variations in susceptibility to lysis by the single buffer used in an analyzer for multiple species.
2. Radiofrequency conductivity: A high-frequency alternating electromagnetic current can be added across the aperture of an impedance analyzer to further characterize cells of similar size. The current penetrates each cell as it passes though the aperture, and the current is altered based on the cell's nuclear and cytoplasmic features including nuclear to cytoplasmic ratio, nuclear density, and cytoplasmic granularity. Impedance and conductivity results are combined to classify the cells.
3. Light scatter (Figure 2.5): As individualized cells in a stream of fluid pass through a beam of laser light, they scatter light in different directions based on cell size, internal structure, cytoplasmic granularity, surface structure, wavelength of light, and fluid medium. The light signals are detected at different angles of scatter and converted to electrical impulses that are used to differentiate the major WBCs by using species-specific algorithms. Abnormal and some normal WBCs may not be correctly classified.
4. Cell absorbance after staining: The light absorbance of WBCs is measured by a light detector in the path of light traveling through each cell. In ADVIA analyzers, after lysis of RBCs, WBCs containing the enzyme myeloperoxidase are stained by adding H_2O_2 and a chromogen substrate (4-chloro-1-naphthol) that generates a black precipitate.
   a. The degree of absorbance varies from none (lymphocytes, basophils, nRBCs, feline eosinophils, blasts) to lesser (monocytes), greater (neutrophils), and greatest (eosinophils of some species) amounts.
   b. This information is combined with volume measurements to classify most WBCs. However, large cells lacking peroxidase staining are called *large unstained cells (LUCs)* and may be large lymphocytes, monocytes, blasts, or basophils.
   c. Because feline eosinophils lack peroxidase activity, they are detected by staining with oxazine 750, a stain also used for detecting reticulocytes.
   d. Eosinophils of many greyhounds and fewer dogs of other breeds have low peroxidase activity and are misclassified as monocytes or LUCs.
   e. Myeloperoxidase content of neutrophils may be decreased with accelerated neutropoiesis, thus merging neutrophil and monocyte populations together and yielding inaccurate neutrophil and monocyte data. This was seen 6 d after endotoxin administration to horses, supporting an effect on early myeloid cells in the bone marrow.[33] However, in other types or stages of inflammation, neutrophil myeloperoxidase content can be increased, and it is often unchanged.
5. Fluorescent labeling (fluorescent flow cytometry): Fluorescent dyes (e.g., polymethine) bind to cell structures and emit specific wavelengths of light when passing through the laser beam of an optical analyzer. The side-emitted fluorescence is detected along with

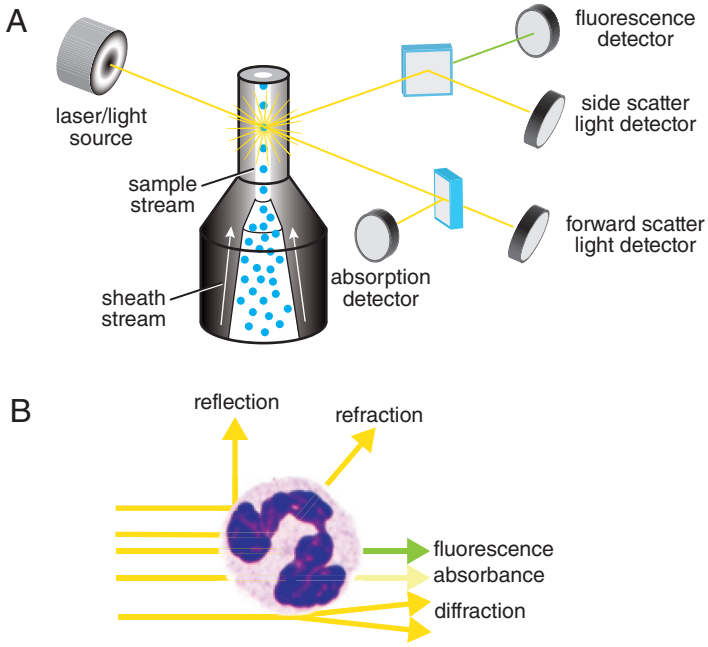


Figure 2.5. [WBC] and WBC identification via flow cytometry methods.

A. Optical scatter, fluorescence, and absorption: Blood cells (*blue dots*) suspended in an isotonic (approximately) diluent are injected into a special flowing fluid. The fluid dynamics in the system create a fluid sheath around the diluent to form a sample stream of mostly single-file cells. Cells in the sample stream pass through a laser or white light beam. Each cell scatters the light in multiple directions, the scatter depending on a cell's size and contents. Lenses, filters, beam splitters, and a forward path obscurance shield (mostly not shown) are used to direct the light to detectors that provide information about each cell.

B. Side scatter is caused mostly by reflection and refraction and generally increases with greater cellular complexity. Forward scatter is caused mostly by diffraction of light at the cell's margin and generally increases with greater cell volume. Additional side-sensors can be used to assess cell absorbance or fluorescence after incubating cells with a dye (e.g., for myeloperoxidase) or fluorescent marker (e.g., for nucleic acids). Computer programs analyze the data from the sensors to determine which cell has passed through the laser beam.

laser side scatter (internal complexity) to help differentiate cells. Fluorochrome-conjugated antibodies targeting surface markers may be used to further characterize WBCs (e.g., lymphocyte subsets).

6. Quantitative buffy coat (QBC) analysis[34,35] (QBC VetAutoread): When blood undergoes centrifugation in a special capillary tube containing a plastic cylinder of an appropriate density, the cylinder floats in the region of the buffy coat and expands the buffy coat and its layers around the float (Figure 2.6). Fluorescence is used to differentiate cell layers on the basis of lipoprotein, RNA, and DNA contents.
   a. If the quantity of blood that is put into the capillary tube is constant and the mean volumes of each cell type are relatively constant, then the concentration of a given cell type correlates with the thickness of the layer for that cell type. Based on these assumptions, the thickness of a layer is multiplied by a conversion factor to obtain an approximate cell concentration. Alterations in WBC densities or staining may result in erroneous results.

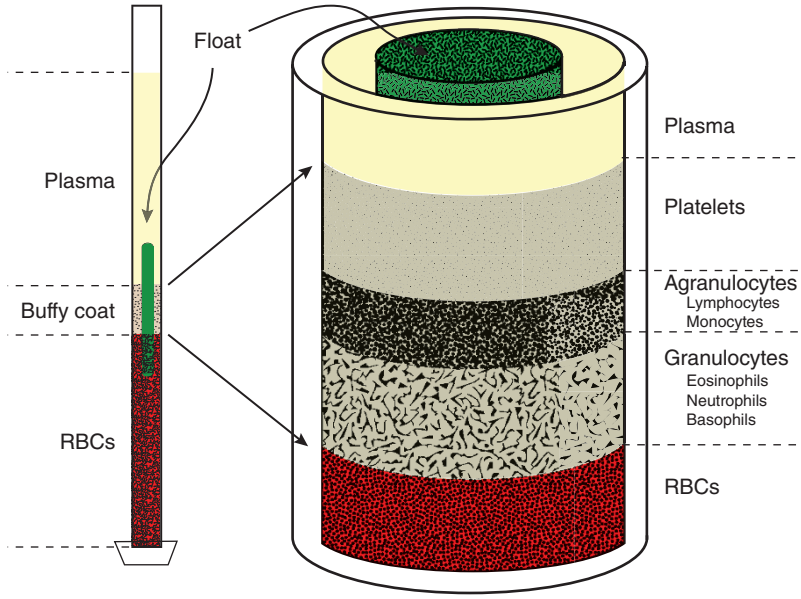


Figure 2.6. Schematic representation of IDEXX QBC VetTube after centrifugation of whole blood. Centrifugal forces separate the components of blood into five layers (plasma, platelets, agranulocytes, granulocytes, and RBCs) based on their relative densities. The buffy coat (composed of platelets and WBCs) is expanded by a float that has a density similar to buffy coat cells. Layers are recognized by use of fluorescence markers for DNA, RNA, and lipoprotein, and the thickness of each cell layer is used to derive cell concentrations (see the text). nRBCs localize to the top of the RBCs and may mask the beginning of the granulocyte layer.

   b. Total [WBC] is calculated by adding the granulocyte and combined lymphocyte/monocyte concentrations. Nucleated RBCs do not interfere with the [WBC].
   c. Granulocyte concentration is determined by the thickness of the cell layer with characteristic DNA and RNA/lipoprotein fluorescence for granulocytes. An eosinophil concentration may be reported for dogs and cattle because their eosinophils show detectably more RNA/lipoprotein fluorescence than do their neutrophils and basophils.
   d. Lymphocyte/monocyte concentration is determined by the thickness of the cell layer above the granulocyte layer that has the DNA fluorescence characteristic of lymphocytes and monocytes.

7. Digital imaging: Dedicated blood smear scanners that display WBC images with their analyzer-assigned classifications have been developed for veterinary hematology samples (CellaVision, Sysmex). WBCs (and nRBCs) are automatically located, imaged, and classified; the images can be reviewed locally or remotely to confirm or alter analyzer preclassifications, which is particularly important in sick animals.[36] Digital imaging is limited by poor smear quality, poor staining, and the automated selection of regions and cells for evaluation; review of images is particularly important for recognizing blasts, basophils, and a variety of dysplastic cells.[37]
8. Combined methods: Variations of the preceding methods are commonly combined in attempts to optimize hematology analyzer utility. Here are a few examples of analyzers used in veterinary medicine.

a. CELL-DYN instruments (Abbott) use impedance methods and optical methods to determine the white cell impedance count (WIC) and the white cell optical count (WOC), respectively. When the WIC and WOC disagree (e.g., when nRBCs are present), an error message is generated by the instrument. WBCs are differentiated by measuring optical light scatter at 0°, 10°, and 90°, and depolarized light scatter at 90°.
b. ADVIA instruments (Siemens) use light scatter combined with absorption to determine a peroxidase channel [WBC], a basophil channel [WBC], and a differential WBC count.
   (1) In a *peroxidase channel,* light scatter is used to count and size WBCs after RBC lysis, and WBCs are assessed for peroxidase content by absorbance as described above.
   (2) In the *basophil channel,* a different lysing agent lyses RBCs and WBCs other than basophils in human samples, and then light scatter is used to differentiate particle sizes (intact human basophils versus other cell nuclei) and nuclear density or lobularity. The basophil channel provides a [WBC] and percentages of minimally lobulated cells (lymphocytes, monocytes, immature granulocytes, and blastic cells), basophils (in human samples), and polymorphonuclear cells (neutrophils and eosinophils).
   (3) Canine and feline basophils are not specifically identified by the basophil or peroxidase channels.
   (4) If lyse-resistant RBCs are present, the [WBC] of the peroxidase channel is falsely increased, but the basophil channel [WBC] should be accurate. The presence of nRBCs is suggested if the "polymorphonuclear cell" concentration from the basophil channel is greater than the sum of the neutrophil and eosinophil concentrations from the peroxidase channel (except in cats; their eosinophils lack peroxidase activity).
c. Sysmex and ProCyte Dx hematology analyzers combine flow cytometry, impedance, and optical fluorescence to generate the quantitative portions of a CBC. The [WBC] is measured by impedance after lysing cells with a surfactant, and the WBC differential is based on fluorescence flow cytometry. Within the analyzer, WBCs are permeabilized and incubated with a nucleic acid stain that penetrates cells and binds to RNA and DNA. Side fluorescence (amount of nucleic acid) and side light scatter (mostly internal complexity) of each WBC passing through a flow cell are used to differentiate them, while forward scatter (cell volume) differentiates the WBCs from RBCs and platelets. In health, monocytes (canine) or eosinophils (feline) have the greatest side fluorescence while neutrophils have the least, and eosinophils have the greatest side scatter of light. Note: The ProCyte One differs from the ProCyte Dx; it differentiates WBCs by multi-angle light scatter without fluorescence.

9. Flags: Hematology analyzers are designed to *flag* (identify) results that may not be accurate, and to generate statements to alert the user and direct action. Efforts are made to limit flagging enough that users are not desensitized to it but not to limit it so much that significant errors occur. However, not all errors are flagged. Flags may also suggest the presence of abnormal WBCs such as blasts, atypical lymphocytes, immature neutrophils, or nRBCs. Appropriate actions in response to flagged results include rerunning the sample and, commonly, evaluating the blood microscopically.
10. Graphical output: Analyzer dot plots (scatter plots) and histograms allow visual inspection of data and recognition of analyzer errors, including potential

misclassification of WBCs, which may or may not generate warning flags.[38] Errors are most likely in sick patients with abnormal WBC concentrations or features. It is good practice to evaluate the graphical output for every sample and further assess samples with atypical patterns; this should include blood smear evaluation by a trained individual.

C. Differential WBC count by microscopy
   1. A microscopic differential WBC count is done by differentiating 100 or more consecutive WBCs on a stained blood film. Results of the differential count are percentages. A differential WBC count should be done in a blood film's optimal zone (counting window). This region is where the WBCs lie flat enough for their nuclear and cytoplasmic features to be distinct, and where RBCs form a monolayer with few touching each other (RBCs are more widely spread in samples from anemic patients). Many smear regions are monolayer areas but not optimal areas for assessing cells because the cells are not flattened adequately (in thicker areas), or they are broken (in thinner areas).
   2. The differential WBC count is only an estimate of WBC percentages in a blood sample because of two major factors:
      a. Sampling error: In the microscopic differential count, only a small percentage of all WBCs in a blood sample are differentiated; the differentiated cells may not be representative. This sampling error can be reduced if more WBCs are included in the differential count, but this process also requires more time, and may be limited by the number of WBCs in optimal regions of a smear (e.g., with decreased [WBC]). As a rule of thumb, the count should differentiate 100 WBCs for every 10,000 WBCs/μL to obtain representative percentages.
      b. Identification accuracy: A skilled laboratory technologist/technician should be able to identify most WBCs accurately during the microscopic examination of mammalian blood, but there are often rare cells that are difficult or impossible to classify, and sometimes a substantial number, e.g., severely toxic neutrophils versus monocytes, or atypical lymphocytes versus monocytes.
   3. Accuracy and precision of the microscopic WBC differential count
      a. Assuming proper sample collection and handling and that cells are identified correctly, the accuracy and precision of the WBC percentages are determined by the number of WBCs included in the differential count and the actual WBC percentage in the blood. A confidence interval table of WBC differential counts has been generated (Table 2.3) that addresses those two factors.[39]
      b. Evaluation of the data in Table 2.3 reveals several key concepts.
         (1) The confidence interval becomes smaller (improves) as the number of cells in the differential count increases. However, it is not practical to do a 1000-cell microscopic differential count in most blood films.
         (2) The confidence intervals are large enough (especially for the 100-cell count) to yield considerable uncertainty in the results (Table 2.4). Thus, the blood of an animal may have exactly the same WBC concentrations on two different days, but the differential WBC counts may differ considerably simply because of encountering different cells during the differential cell counts.
      c. The calculated concentration of each WBC type is the appropriate value to interpret, not the differential count percentage. Some people refer to concentrations as "absolute counts" and percentages as "relative counts;" both are estimates for most CBC results.

Table 2.3. The 95 % confidence limits[a] for percentages obtained in differential WBC counts[b,c]

	Number differentiated[d]				Number differentiated		
α[e]	100	200	1000	α	100	200	1000
0	0–4	0–2	0–1	10	4–18	6–16	8–13
1	0–6	0–4	0–2	15	8–24	10–21	12–18
2	0–8	0–6	1–4	20	12–30	14–27	17–23
3	0–9	1–7	2–5	25	16–35	19–32	22–28
4	1–10	1–8	2–6	30	21–40	23–37	27–33
5	1–12	2–10	3–7	35	25–46	28–43	32–39
6	2–13	3–11	4–8	40	30–51	33–48	36–44
7	2–14	3–12	5–9	45	35–56	38–53	41–49
8	3–16	4–13	6–10	50	39–61	42–58	46–54
9	4–17	5–15	7–11	80	70–88	73–86	77–83

[a] Confidence intervals for the actual percentage of a given WBC if the specified number of cells were differentiated and the observed percentage was α

[b] Source: Rümke[23]

[c] If observed percentage *x* is greater than 50 %, then confidence limits are obtained by subtracting the confidence limits of (100 – *x*) from 100. For example, if *x* = 80 % in a 100-cell count, then the confidence limits for 20 (100 – *x*) are subtracted from 100 (100 – 30 or 100 – 12) to give reference limits of 70 % and 88 %, respectively.

[d] Number (100, 200, or 1000) of WBCs differentiated in a blood film

[e] α is the percentage of a given WBC found in a differential count.

Table 2.4. Examples of [WBC] differences due to random error of microscopic differential WBC counts if the total [WBC] is 10.0 $\times 10^3/\mu L$

	Actual values		Confidence limits		RI
	%	$\times 10^3/\mu L$	%[a]	$\times 10^3/\mu L$	$\times 10^3/\mu L$
Band neutrophils	0	0.0	0–4	0.0–0.4[b]	0.0–0.1
Band neutrophils	4	0.4	1–10	0.1–1.0[c]	0.0–0.1
Eosinophils	15	1.5	8–24	0.8–2.4[d]	0.0–1.3

[a] Confidence limits percentages obtained from Table 2.3 (100-cell WBC differential count)

[b] Based on confidence limits and a 100-cell differential count yielding 0 % band neutrophils, a mild left shift (bands as high as 400/μL) could be present, but it is not in this case.

[c] A mild left shift may or may not be detected when it is present. In this example, 1 % band neutrophils is within the 95 % confidence limits for a 100-WBC differential count of a sample with 4 % band neutrophils, and that would reflect a [neutrophil] of 0.1 $\times 10^3/\mu L$, which is WRI.

[d] Mild eosinophilia may not be detected when present, e.g., if another differential count yielded 8–13 % eosinophils, all within the 95 % confidence limits.

D. Correction of [WBC] for the presence of nucleated RBCs (nRBCs)

1. Microscopic and some electronic methods determine [WBC] by counting nucleated cells or nuclei (either of WBCs or nRBCs). By these methods, a measured [WBC] represents the sum of the concentrations of WBC and any nRBCs that were detected.

2. If nRBCs are observed during the examination of the blood film, they are enumerated by counting the number of nRBCs seen while 100 WBCs are differentiated and counted. The conventional method of recording the number of nucleated RBCs is the number of nRBCs for every 100 WBCs (e.g., 10 nRBCs/100 WBCs).
3. Rule of thumb: If there are 10 or more nRBCs/100 WBCs, the measured [WBC] is corrected for the presence of nRBCs. The error in the measured [WBC] is usually not clinically significant when there are fewer than 10 nRBCs/100 WBCs. However, the correction may be done with fewer than 10 nRBCs/100 WBCs.
4. The corrected [WBC] equation (Eq. 2.2) assumes that all nRBCs were detected by the analyzer and included in the analyzer's [WBC].

$$\text{corrected [WBC]} = \text{measured [WBC]} \times \frac{100}{100 + \#\ \text{nRBC}/100\text{WBC}}$$

$$\text{Or, corrected [WBC]} = \text{measured [WBC]} \times \frac{100}{100 + \text{nRBC}},$$

where nRBC is the number of nRBCs seen per 100 WBCs (**2.2**)

Example: measured [WBC] = 20,000/μL, nRBC = 25/100 WBC

$$\text{corrected [WBC]} = 20{,}000/\mu\text{L} \times \frac{100}{100 + 25} = 20{,}000/\mu\text{L} \times \frac{100}{125} = 16{,}000/\mu\text{L}$$

   a. While 100 WBCs were counted, 25 nRBCs were also counted. Thus, there were actually 100 WBCs out of a total of 125 nucleated cells. Therefore, 100 of 125 nucleated cells (4 of 5) must be WBCs.
   b. If the measured [WBC] was 20.0 $\times 10^3/\mu$L, then 4 of 5 of the nucleated cells were WBCs, and thus the corrected [WBC] is 16.0 $\times 10^3/\mu$L.
5. The concentrations of the individual types of WBCs are calculated from their microscopic percentages and the corrected [WBC]; these are the results that are interpreted. When nRBCs are a large proportion of a sample's nucleated cells, the corrected estimates of WBC concentrations can substantially and appropriately alter interpretation of the leukogram. However, the added imprecision caused by nRBC enumeration in addition to WBC enumeration should be considered when interpreting the corrected results (Table 2.3).

E. Nonstaining eosinophil granules (grey eosinophils) of dogs[40–43] and cats[44]
   1. Canine eosinophils typically contain numerous round eosinophilic granules of varying diameters, along with a few cytoplasmic "vacuoles." In individuals of some breeds (especially greyhounds, Italian greyhounds, and whippets but also borzois, deerhounds, and sporadically golden retrievers, Shetland sheepdogs, and other breeds), all the eosinophils contain nonstaining granules that look like cytoplasmic vacuoles, sometimes with central foci (cores) of eosinophilic staining. The cytoplasm of these canine eosinophils sometimes appears grey, thus the name *grey eosinophils*. Altered granule staining represents a modified chemical composition; the altered composition is associated with ultrastructural changes despite publications to the contrary (author's unpublished data, Figure 2.7). Some greyhounds have eosinophils with nonstaining granules and decreased peroxidase activity, few greyhounds have granules that stain but lack peroxidase activity, and some greyhounds lack any eosinophil granule abnormality (author's unpublished findings). The same patterns occur in dogs of other breeds. Therefore, reduced staining and reduced eosinophil peroxidase content are often concurrent, but reduced peroxidase content can occur without abnormal staining.

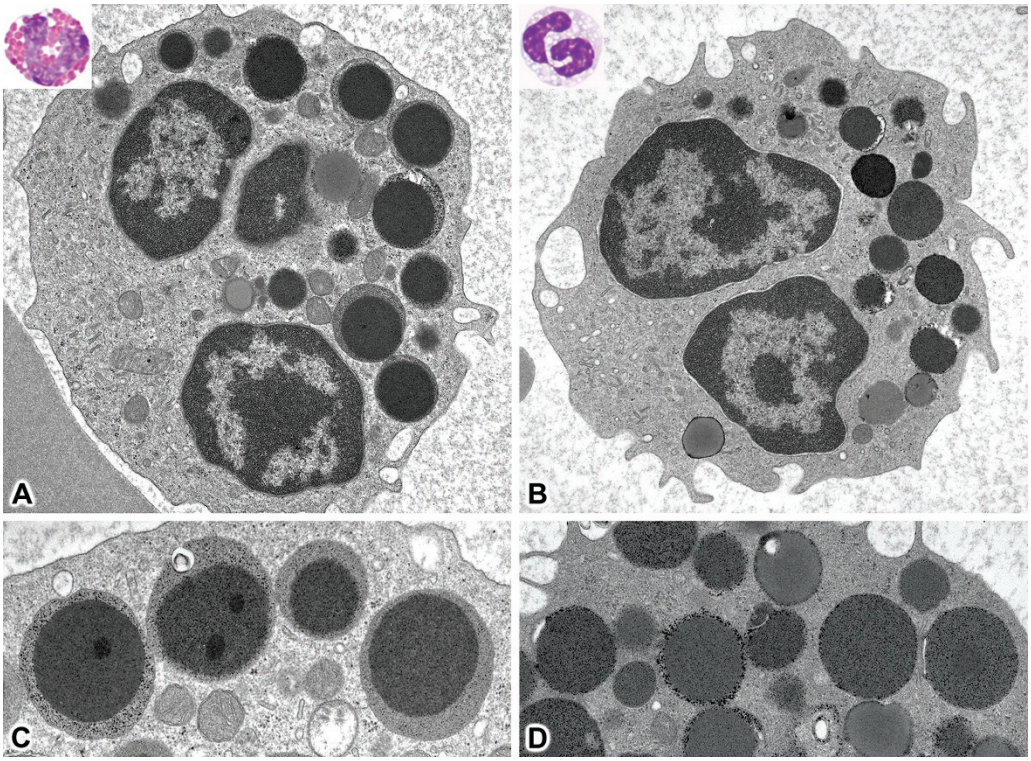


Figure 2.7. Transmission electron micrographs (TEMs) of typical nongreyhound (NGr) eosinophils (A and C) and greyhound (Gr) eosinophils with nonstaining granules (B and D). Paired lower (A and B) and higher (C and D) magnifications are equal. The Gr specific granules are generally smaller with thinner outer rims around smaller areas of dense matrix. Analysis of 114 Gr and 108 NGr eosinophil granules in TEMs of five eosinophils from each of three Grs and three NGrs showed that the mean maximum width of Gr granules was 80 % that of NGr granules, the mean maximum width of the dense matrix was 88 % that of NGrs, and the mean outer rim width calculated as the difference between these two values was 55 % that of NGrs. Also, 24 % of the Gr granules had rim irregularities compared to only 3 % of the NGr granules, and the outer rims were darker than the dense matrix in 46 % of the Gr granules compared to 10 % for NGrs. Dense cores were present in 4 % of Gr granules compared to 14 % of NGr granules (three are apparent in frame C). All these differences were statistically significant. Dr. Nora Berghoff analyzed the cells; Donna Craft and Ralph Common produced the TEMs, Electron Microscopy Laboratory, Michigan State University.

2. Eosinophils in rare cats are packed with nonstaining round granules instead of the typical eosinophilic round to rod-shaped granules.[44] These granules did not stain with Luna or Luxol fast blue stains and lacked dense cores when assessed by transmission electron microscopy. All feline eosinophils lack eosinophil peroxidase.
3. There currently is no known clinical or pathologic significance to the presence of these eosinophils other than the potential for incorrectly identifying the cells in hematologic, cytologic, or histologic samples.

a. Microscopically, they are sometimes incorrectly identified as toxic neutrophils or monocytes. However, the cytoplasmic foaminess in neutrophils with toxic change is caused by cytoplasmic clearing, which has a different appearance from distinct vacuolation, and other toxic changes would be expected. Monocyte vacuolation is typically sparce or heterogeneous, and monocyte nuclei typically are broader with less condensed chromatin than granulocytes.
b. Automated analyzers using various methods may misclassify them or underestimate their concentration because of decreased myeloperoxidase, altered staining, or altered refraction of light.[42,43]
c. Lack of eosin staining also impairs detection of these eosinophils in tissue sections routinely stained with hematoxylin and eosin, but canine eosinophil granules can be identified by Luxol fast blue staining.[45]

F. Leukoagglutination
1. Occasionally, aggregates of 3–15 or even dozens of WBCs are found within the body of a well-prepared blood film. Typically, they are mixtures of mostly neutrophils or small lymphocytes with fewer eosinophils and monocytes. They should be suspected when WBCs are distributed in relatively dense streaks at the transition between the optimal zone and the feathered end of the blood film, running parallel with the long axis of the smear. These streaks are aggregates that have been disrupted. The body and the thick end of the smear should be inspected to confirm leukoagglutination.
2. Published studies of WBC aggregation are restricted to human samples. They show that aggregation of neutrophils is typically an artifact of anticoagulation and is dependent on the presence of EDTA and often on temperature. The aggregation may also occur in citrated or heparinized samples.[46,47] When aggregation was seen in room-temperature samples but not in samples kept at 37 °C, the neutrophil aggregation was due to an IgM.[48]
3. A major reason for recognizing WBC agglutination is that the measured WBC concentrations can be erroneous.[49] The aggregated WBCs may not be enumerated correctly by the instruments that use impedance or laser methods if the WBCs do not break apart in the diluent, and thus the total WBC concentration would be lower than the true value (i.e., pseudoleukopenia). The aggregated WBCs may be poorly distributed in a blood film, and thus the differential WBC count may be inaccurate if one type of WBC is preferentially aggregated.
4. WBC aggregates have been seen in canine, feline, and equine blood; in some of these samples (authors' observation), the aggregation was EDTA dependent (i.e., seen in EDTA samples but little to none seen in citrate samples). Aggregation increased with lower sample temperature and with increased storage time. The authors are not aware of published investigations of leukoagglutination in samples from domestic mammals.

III. Relative versus absolute changes in leukogram results
A. Absolute changes: Except with the centrifugation method, WBC concentrations are calculated by multiplying the total WBC concentration by the percentages obtained from the differential count. Changes in these calculated concentrations are often called *absolute changes* in the leukogram.
B. Relative changes: A change in the percentage of an individual WBC type is commonly called a *relative change*, but it may not reflect a true change in that WBC type.
1. As shown in Table 2.5, detection of true changes in a WBC population should be based on evaluation of the calculated *concentrations* of the individual WBC types.

Table 2.5. WBC percentages versus WBC concentrations

	Units	Dogs				RI
		1[a]	2[b]	3[c]	4[d]	
Neutrophils	%	30	30	70	70	60–80
Lymphocytes	%	70	70	30	30	20–35
WBCs	$\times 10^3/\mu L$	2.0	50.0	2.0	50.0	6.0–17.0
Neutrophils	$\times 10^3/\mu L$	0.6	15.0	1.4	35.0	3.0–11.5
Lymphocytes	$\times 10^3/\mu L$	1.4	35.0	0.6	15.0	1.0–4.0

[a] Dog 1: Although the percentage of lymphocytes is increased, the lymphocyte concentration is WRI and there is not a lymphocytosis because the [WBC] is low (a high percentage of a low number is a low number). Neutropenia is present.
[b] Dog 2: Although the percentage of neutrophils is decreased, the neutrophil concentration is increased and there is a mild neutrophilia because the [WBC] is relatively high (a low percentage of a large number may be a large number). Lymphocytosis is also present.
[c] Dog 3's WBC percentages are WRIs, but neutropenia and lymphopenia are present (the [WBC] is low).
[d] Dog 4's WBC percentages are WRIs, but neutrophilia and lymphocytosis are present (the [WBC] is high).

2. If a WBC percentage is increased or decreased compared to RIs, it may be because of changes in that cell type or because of changes in the total [WBC]. For example, dog 2 in Table 2.5 has a decreased neutrophil percentage, but the results actually indicate the dog has a neutrophilia (the neutrophil concentration is above the URL). When interpreting dog 2's CBC results, one should consider potential causes of neutrophilia, not neutropenia.

C. Changes in WBC percentages should not be considered when interpreting a leukogram. Throughout this book, terms used to describe leukogram findings refer to changes in cell concentrations, not changes in percentages.

ABNORMAL WBC CONCENTRATIONS IN BLOOD

Leukocytosis is an increased [WBC] and *leukopenia* is a decreased [WBC]. The significance of these abnormalities depends on the specific type(s) of WBCs responsible for them, and thus the concentrations of each WBC type must be evaluated individually and with respect to patterns. Terms used when the concentration of each WBC type exceeds its URL are *neutrophilia*, *lymphocytosis*, *monocytosis*, *eosinophilia*, and *basophilia*. Terms used when the concentration of each WBC type is below its LRL are *neutropenia*, *lymphopenia*, *monocytopenia*, *eosinopenia*, and *basopenia*. In publications, it is not uncommon for these terms to be used for increases or decreases in values of one group compared to another group, even if values remain WRIs.

I. Abnormal neutrophil concentrations
   A. Left shift
      1. A *left shift* (shift to the left, bandemia, band neutrophilia) is an increased concentration of nonsegmented neutrophils (usually bands) in blood. When inflammatory stimuli are great enough, younger stages (metamyelocytes, myelocytes, and rarely progranulocytes) also may be present in the left shift.

a. The term *left shift* has been attributed to Josef Arneth, who, in the early 1900s, tabulated neutrophils by their nuclear lobulation, with the most immature form (Class 1) in the left column and the most lobulated form (Class 5) in the right column.[51,52] He referred to a shift of the blood picture to the left ("Verschiebung des Blutbildes nach links," p.32) when numbers increased in the leftward columns of the table and decreased in columns to the right.
b. A *band neutrophil* (not a banded neutrophil) is an immature neutrophil with a band-shaped nucleus, meaning the nucleus has nearly parallel sides without clear segmentation or substantial indentations (in the shape of a C, an S, or a U),[50] and it is most often broader with less mature chromatin (paler, less clumped) than segmented neutrophils in the same sample. However, the distinction between band neutrophils and segmented neutrophils can be unclear for several reasons including: (i) the entire shape of a neutrophil's nucleus may not be discernible (e.g., the nucleus folds back over itself, (ii) maturation occurs along a spectrum, creating questions of when indentations are substantial enough to classify cells as segmented neutrophils, and (iii) maturation can be altered in response to disease or drugs (see toxic neutrophils).
   (1) Although universal application of appropriate neutrophil classification criteria would be desirable, various approaches are encountered when classification is unclear. Approaches for classifying neutrophils that have parallel sides where assessable but nuclear shapes (folds) that might mask indentation include:
      (a) Using the adage that "when in doubt, call it the older cell"
      (b) Labeling them band neutrophils if their visible nuclear features are immature and as segmented neutrophils if visible nuclear features are mature (i.e., using chromatin and nuclear width as secondary criteria)
   (2) Laboratories may attempt to minimize intralaboratory differences, but care should be taken not to overinterpret mild changes in band neutrophil concentration, even from the same laboratory.
   (3) Some automated hematology analyzers provide a measure of neutrophil or granulocyte immaturity or report when immature forms are suspected; microscopic evaluation should be used to confirm, refute, or further characterize the potential immaturity. (The immature granulocyte assessment used in some human hematology analyzers is limited to metamyelocyte, myelocytes, and promyelocytes.)
c. Left shifts occur when release of neutrophils from marrow diminishes the NSP, and younger cells are then released from the NMP. This may relate to decreased competition from mature cells for egress-promoting chemokines, or to cytokine-induced decreases in expression of cell retention factors or their receptors on immature neutrophils. Left shifts occur sooner in species or individuals with smaller NSPs.
d. Because it usually occurs in response to relatively intense, often acute inflammatory stimuli, a left shift is frequently considered the hallmark of acute inflammation. Such inflammation is typically caused by infectious agents (e.g., pyogenic bacteria, fungi) but can be caused by noninfectious disorders (e.g., necrosis, immune-mediated disease, or neoplasms). Glucocorticoid hormones and endotoxins also stimulate release of neutrophils and thus may cause mild left shifts.

2. Left-shift classifications
   a. Severity: Two features might be considered when describing the severity of a left shift. Since the terms can describe different findings, one must be careful in interpreting or using them.

(1) Immaturity of neutrophils in the left shift: bands only (1+ or slight); band and metamyelocytes (2+ or moderate); and band, metamyelocytes, and myelocytes (3+ or marked)[53]

(2) Magnitude of nonsegmented neutrophil concentrations: Universal numeric cutoffs are lacking, but < 1.0–2.0 $\times 10^3/\mu L$ is mild, > 10.0 $\times 10^3/\mu L$ is definitely marked, and in between is moderate. Marked left shifts are not common in adult ruminants, horses, alpacas, and llamas. Inflammation may be marked when left shifts are mild, moderate, or marked.

b. Regenerative versus degenerative left-shift classifications

(1) Although definitions are inconsistent, the clinical significance of these classifications is generally similar across definitions.

(a) A regenerative left shift suggests a more favorable inflammatory state in which, at the time of sampling, neutrophil production and release are adequately responding to the demand.

(b) A degenerative left shift suggests a less favorable inflammatory state in which neutrophil production and release are not adequately responding to the demand. Band neutrophils are substantially less capable of bacterial killing and chemotaxis than segmented neutrophils (less mature forms being even less functional),[54] so a smaller percentage of neutrophils are optimally functional when a degenerative left shift is present.

(2) From the recent history of these terms outlined below, a left shift with a predominance of immature neutrophils is a *degenerative left shift*, and a left shift with a neutrophilia and predominance of mature neutrophils is a *regenerative left shift*. Historical statements did not address application of the terms to some leukogram patterns, e.g., when a [neutrophil] is decreased or WRI and mature neutrophils outnumber immature ones. However, a left shift in a neutropenic patient is generally unfavorable whereas a mild left shift with a [neutrophil] WRI may or may not be unfavorable. Serial CBC data often clarify the neutrophil response.

(3) In the first three editions of *Veterinary Hematology*, Dr. O.W. Schalm attempted to provide guidance in classifying left shifts. In *Schalm's Veterinary Hematology* (fourth edition), Dr. Jain described these states as follows:[55]

(a) "Regenerative left shift is characterized by a leukocytosis due to neutrophilia and with the appearance of immature neutrophils in peripheral blood. … In the typical regenerative left shift, the proportion of various immature neutrophils is orderly and follows a pyramidal distribution, with the most immature cell being the least numerous. Usually, the mature neutrophils outnumber the immature cells" (p. 824).

(b) "The main feature of a degenerative left shift is the occurrence of young neutrophilic granulocytes in the circulation in numbers exceeding mature neutrophils. The WBC count is often suggestive of leukopenia, but it may sometimes be within the normal range or, rarely, elevated" (p. 824).

B. A *right shift* (shift to the right) is an increased concentration of hypersegmented neutrophils in blood, which in most of our domestic mammals, are considered to be neutrophils with five or more nuclear lobes. A criterion of seven or more lobes has been suggested for horses because equine neutrophils have irregular nuclear margins (jagged nuclei) with a greater average number of nuclear segments – five in one small study.[56] Hypersegmented neutrophils

without atypia usually are thought to indicate older cells that have aged in blood because of increased circulation time. The presence of hypersegmented neutrophils in blood is typically recorded as a comment in the CBC results and not as a separate part of a WBC differential count (if it is noted at all).

1. Glucocorticoid hormones (endogenous or exogenous) may decrease emigration of neutrophils to tissue by down-regulating adhesion molecules or decrease neutrophil apoptosis and therefore removal from circulation, thus causing a right shift. Similarly, hypersegmentation may be present in patients with LAD.[57,58]
2. Hypersegmented neutrophils may be present in chronic inflammatory diseases, particularly during resolution when there is less stimulus for transendothelial migration (e.g., after hysterectomy for a pyometra when the bone marrow is hyperplastic, but the inflammatory focus has been removed).
3. Atypical, dysplastic, and neoplastic hypersegmentation are discussed later in this chapter (see Abnormal Morphologic Features of WBCs, Section VII. Nuclear hypersegmentation unrelated to cell aging).

C. Neutrophilia (increased measured blood neutrophil concentration) (Table 2.6)

1. Note that neutrophilia is an increased concentration of all neutrophils, not just segmented neutrophils. The segmented neutrophil concentration may be WRI, but the combined segmented and band neutrophil concentrations may exceed the sum of their respective URLs, thus indicating neutrophilia. The term neutrophilia is sometimes used to describe an increase in neutrophils in other tissues or wash fluids.
2. Acute inflammatory neutrophilia

Table 2.6. Disorders and conditions that cause neutrophilia

Inflammation (acute and chronic)
Infections:* bacterial, fungal, viral, protozoal
Immune-mediated hemolytic anemia*
Necrosis:* hemolysis, hemorrhage, burns, neoplasms, infarcts, trauma, sterile inflammation
Neoplasms (via release of inflammatory mediators in addition to necrosis)
Sterile foreign body/material
Increased glucocorticoids (steroid or "stress" neutrophilia)
Stress (physical or neurogenic)*
Hyperadrenocorticism
Glucocorticoid therapy*
Adrenocorticotropic hormone administration
Increased catecholamines (physiologic neutrophilia)
Fight-or-flight response:* excitement, fright, pain, exercise, anxiety
Catecholamine injections: epinephrine or norepinephrine
Chronic myeloid leukemia and chronic neutrophilic leukemia
Neoplasms (paraneoplastic neutrophilia)
Leukocyte adhesion deficiency, type I and III
Others or unknown mechanisms
G-CSF administration
Estrogen toxicosis (early)

* A relatively common disorder, condition, or mechanism

a. This neutrophilia results from changes in neutrophil kinetics that are caused by acute inflammatory mediators. The net result is an increased CNP that may contain a left shift (Figure 2.8B). The pattern may persist chronically (see next section).
   (1) Release from the NSP occurs within minutes after the onset of inflammation and causes initial neutrophilia if release exceeds neutrophil emigration to inflamed tissue.
   (2) Release from the NMP causes a left shift and occurs mostly after reduction or depletion of the NSP.
   (3) Increased production from the myelocyte stage: It takes 2–4 d before effects are seen in systemic blood, the more rapid responses occurring with greater cytokine stimulation to accelerate maturation and release.
   (4) Increased production via stem cells: This leads to granulocytic hyperplasia. It takes about 5 d before effects are seen in blood.
b. Inflammatory mediators must enter systemic blood and stimulate marrow cells for neutrophilia to develop.
   (1) Neutrophilia is expected if there is substantial acute inflammation of subcutaneous or many more internal tissues (respiratory tract, pancreas, peritoneal or pleural cavity, and occasionally uterus, liver, or intestine), because mediators can easily access systemic blood.
   (2) Inflammation of brain, spinal cord, superficial cutaneous lesions, and lower urinary tract may not cause neutrophilia, because mediators are lost (to urine or skin) or do not leave the protected environment (brain or spinal cord).
c. The magnitude of inflammatory neutrophilia varies among species and by age in some species, thus interpretation of specific concentrations may vary accordingly. The purpose of Table 2.7 is to emphasize the need for species-related interpretation of a [neutrophil]. For example, a [neutrophil] of 20.0 $\times 10^3/\mu L$ is commonly caused by inflammation in dogs and cats, but the same [neutrophil] is not commonly caused by inflammation in adult cattle and horses. The data in Table 2.7 are provided as interpretive guidelines, not as decision limits.
d. Inflammatory neutrophilias in adult cattle result primarily from increased production (granulocytic hyperplasia) and not from release of stored neutrophils, because cattle have a small NSP. Neutropenia is common with acute inflammation in cattle.
e. The term *leukemoid response* has been used to describe any extreme leukocytosis (including a marked neutrophilia) that is leukemia-like but not leukemia. By this definition, the term can be appropriately applied only retrospectively; its use for a leukocytosis that proves to be leukemia is inappropriate because the leukocytosis was caused by leukemia, not a leukemia-like (i.e., leukemoid) inflammatory process.
   (1) Disorders associated with a marked inflammatory neutrophilia included focal suppurative lesions (e.g., canine pyometra, pleuritis or pyothorax, peritonitis, prostatitis, pneumonia, and abscesses) and hemolytic anemia (e.g., immune-mediated, babesiosis). Canine hepatozoonosis caused by *Hepatozoon americanum* is another cause.
   (2) Paraneoplastic neutrophilia and the neutrophilia of LAD can also be marked and therefore leukemoid (see later in this section).
f. An animal that has an acute inflammatory neutrophilia usually has a lymphopenia, often an eosinopenia or low-normal [eosinophil], and sometimes a monocytosis or toxic neutrophils. These changes can all be caused by inflammation, but endogenous glucocorticoids may be causative or contributory to all but the toxic neutrophils. In dogs, mastocytemia may be present.

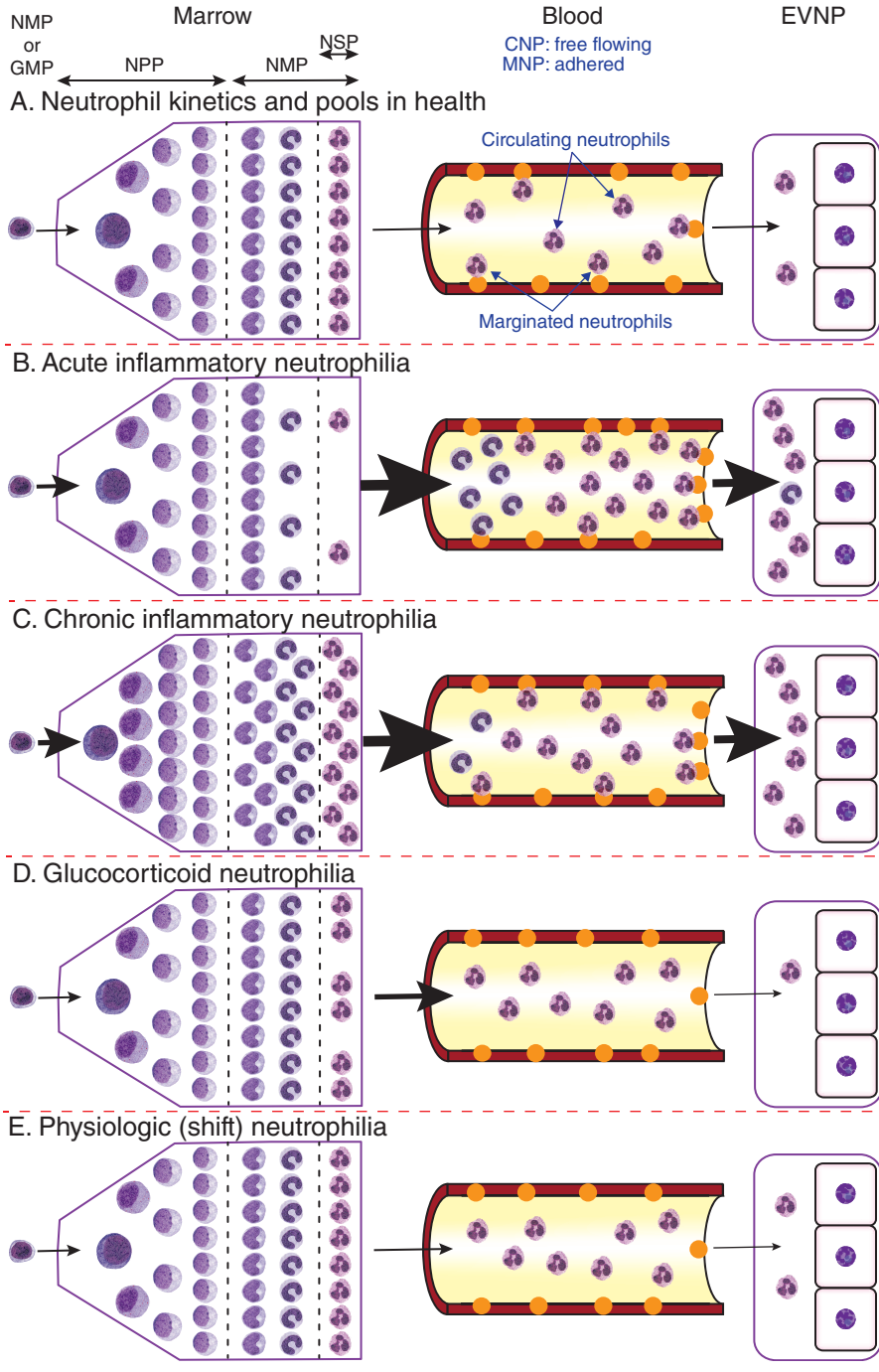


Figure 2.8. Neutrophilia kinetics.

A. Neutrophil kinetics in health (a reduced version of Figure 2.2).

B. Acute inflammatory neutrophilia: Neutrophilia occurs because the release of neutrophils from the marrow exceeds the migration of neutrophils to the inflamed tissue. A left shift is created by the release of band neutrophils from the NMP.

Table 2.7. Species differences in approximate magnitude of inflammatory neutrophilia (adults)

	RI[a]	Common	Uncommon
Dogs	3.0–10.0[b]	< 35.0	> 60.0
Cats	2.0–12.0	< 25.0	> 40.0
Horses	2.0–7.0	< 15.0	> 30.0
Cattle	2.0–7.0	< 10.0	> 20.0
Alpacas	4.0–14.0	< 20.0	> 25.0

[a] Approximate total neutrophil RIs based on several sources
[b] Neutrophil concentration units: $\times 10^3/\mu L$
Note: Neutrophilias of magnitudes between guidelines for common (the majority of cases) and uncommon (small minority of cases) occur with low to moderate frequency.

3. Chronic inflammatory neutrophilia
   a. This neutrophilia results from changes in neutrophil kinetics when an inflammatory process continues for at least a week during which inflammatory mediators stimulate development of granulocytic hyperplasia (Figure 2.8C). In this scenario, the inflammatory stimulus is not so severe that migration out of blood is greater than the bone marrow's capacity to provide neutrophils. If the NSP is replenished, segmented neutrophils are released instead of band neutrophils, and thus the left shift diminishes. In addition, other defense mechanisms become active, and thus the need for neutrophils in tissues may diminish. However, if the inflammatory stimulus is more intense, a left shift may persist, or a chronic inflammatory pattern may never be reached. In these disorders, there is increased production of neutrophils because of granulocytic hyperplasia and increased neutrophil release from the NSP in response to inflammatory mediators. When the rate of neutrophil release from marrow is greater than the rate of neutrophil margination and emigration to tissues, neutrophilia develops.
   b. When an animal has a chronic inflammatory neutrophilia, other WBC abnormalities may include lymphocytosis (with or without reactive lymphocytes), lymphopenia, monocytosis, eosinophilia, basophilia, a left shift, a right shift, or toxic neutrophils. Chronic inflammation may also induce mild anemia.
4. Acute versus chronic inflammatory neutrophilia: Acute and chronic inflammatory neutrophilia patterns form a continuum and can yield similar or identical leukogram patterns. Although clues regarding duration of the inflammation may be present (see above for concurrent changes that may be present with each), differentiating short-duration from long-duration inflammation from only a CBC's leukogram is often impossible. Bone marrow is usually not assessed in neutrophilic patients, so the state of

Figure 2.8. *continued*
C. Chronic inflammatory neutrophilia: Neutrophilia occurs because the release of neutrophils from the marrow exceeds the migration of neutrophils to the inflamed tissue. A left shift may be mild or absent because granulocytic hyperplasia maintains the NSP.
D. Glucocorticoid ("stress") neutrophilia: Neutrophilia occurs because of a shift of neutrophils from the MNP to the CNP, decreased migration of neutrophils to tissue, decreased apoptosis and clearance of mature neutrophils (not shown), and release of neutrophils from the NSP and sometimes the NMP (minimal).
E. Physiologic (shift) neutrophilia: Neutrophilia occurs because of the shift of neutrophils to the CNP from the MNP within the spleen, lungs, and other tissues.

the storage pool and degree of myelopoiesis are usually not known. Clinical history or previous CBCs may suggest or indicate chronicity, but a more realistic goal is just to recognize evidence of inflammation.

5. Increased glucocorticoids (steroid or "stress" neutrophilia)
   a. This neutrophilia results from changes created by the effects of endogenous or exogenous glucocorticoids on neutrophil kinetics (Figure 2.8D). Although this neutrophilia is frequently called a *stress neutrophilia*, it should not be confused with the *physiologic (shift) neutrophilia* caused by the stress-induced release of catecholamines.
      (1) Neutrophils shift from the MNP to the CNP because of decreased expression of neutrophil selectin (for cell rolling) and endothelial cell adhesion molecules (for firm adhesion). Rabbit studies indicated that the decrease in selectin expression occurs for neutrophils within the bone marrow maturation pool rather than those in circulation.[59]
         (a) This process can double the measured neutrophil concentrations in canine, equine, and bovine blood. Greater increases in measured neutrophil concentrations may occur in feline blood because of the larger feline MNP.[7]
         (b) Because of this shift, fewer neutrophils emigrate to tissues, and thus neutrophils have an increased circulating life span. The older neutrophils may become hypersegmented (right shift).
      (2) Apoptosis of mature neutrophils is decreased, which contributes to prolonged survival in circulation and therefore to neutrophilia.
      (3) The transit time through the bone marrow maturation pool is decreased, and release of neutrophils from marrow is mildly increased. Mostly segmented neutrophils are released, but glucocorticoids may cause release of band neutrophils and thus a mild left shift (usually < 1000/μL but it may be more with pre-existing inflammation).[60,61]
   b. Classic steroid leukograms are seen most frequently in dogs, where they consist of neutrophilia (2–4× URL) with little or no left shift (band neutrophils < 1000/μL), lymphopenia, monocytosis, and eosinopenia or low-normal [eosinophil].[60] Inflammation can cause an identical pattern, but the presence of toxic neutrophils would support inflammation. The magnitude of neutrophilia varies with different glucocorticoids and dosages; dogs given anti-inflammatory doses of prednisone (1 mg/kg q24h) had only mild leukogram changes (rarely outside RIs) after 7 and 14 d of treatment.[62] The effects on neutrophil kinetics diminish with chronic elevations in glucocorticoids (e.g., hyperadrenocorticism).
   c. Typical glucocorticoid effects differ among species of animals, particularly with regard to monocytes (Table 2.8).
6. Increased catecholamines (physiologic neutrophilia)
   a. This neutrophilia results from effects of catecholamines (typically associated with fear, excitement, and exercise) that cause a shift from the MNP, including spleen and lungs, to the CNP (Figure 2.8E). The shift is associated with increased blood flow rate, especially for neutrophil movement out of lungs.[64] Neutrophil adherence to endothelial cells may also be reduced.[65]
   b. The magnitude of neutrophilia may be up to about twice the URL for canine, equine, and bovine blood, and potentially up to 3–4× URL for a cat because of a cat's larger MNP.[7]
   c. It is seen most frequently in healthy animals and mostly in cats. WBC concentrations return to RIs relatively fast (within an hour) if the stimulus disappears.

Table 2.8. Expected WBC concentrations in animals with glucocorticoid leukograms

WBC concentrations	Dogs	Cats	Horses	Cattle	Camelids[a]
Total WBCs ($\times 10^3/\mu L$)	15.0–35.0	15.0–30.0	10.0–20.0	8.0–18.0	—
Segmented neutrophil[b]	↑	↑	↑	WRI–↑	—
Band neutrophil[b]	WRI–↑[c]	WRI	WRI–↑[c]	WRI	—
Lymphocyte[b]	↓	↓	↓	↓	—
Monocyte[b]	WRI–↑	WRI–↑	WRI	WRI–↑	—
Eosinophil[b]	↓–WRI[d]	↓–WRI[d]	↓–WRI[d]	↓–WRI[d]	—

[a] Data not found; "stress-induced leukocytosis," which could involve catecholamines, reportedly can exceed 40,000/μL with substantial increases in [band neutrophils][63]

[b] Presence or absence of abnormal WBC concentrations are based on statements in respective chapters of *Schalm's Veterinary Hematology*, 4th ed.[55] and authors' experiences.

[c] Left shifts are mild when present.

[d] Expected to be decreased or low-normal; decreased eosinophil concentrations may not be detected by routine CBC results because of the imprecision of WBC differential counts, and because the LRL may be 0/μL (so decreases cannot occur)

   d. Because the increased blood flow rate alters kinetics of other WBCs, there also may be increased concentrations of other WBCs, especially lymphocytes, which are typically the other most numerous WBC type in the blood.

7. Chronic myeloid leukemia and chronic neutrophilic leukemia (Table 6.5)
   a. In these leukemias, neutrophilia is caused by a clonal proliferation of neutrophils that are well-differentiated enough to be classified as neutrophils; these conditions are difficult to differentiate from nonneoplastic neutrophilias. There may be an extreme leukocytosis with a left shift that is more apparent in the bone marrow.
   b. With acute myeloid leukemia, the cells would include many immature granulocyte precursors that would not be classified as neutrophils in leukogram results.
8. Neoplasms causing paraneoplastic neutrophilia
   a. Neoplastic tissues may produce G-CSF, GM-CSF, IL-6, or a similar substance that stimulates neutropoiesis. This direct stimulation of neutropoiesis is different from stimulation caused by inflammation secondary to a neoplasm. The neutrophil concentrations of the blood may resemble either an inflammatory neutrophilia (variable degrees of left shift that may be accompanied by monocytosis) or chronic leukemia. Although not definitive, the conclusion that a paraneoplastic leukocytosis is present is most commonly based on the association of a marked leukocytosis with a neoplasm, exclusion of other causes of the leukocytosis, and resolution of the leukocytosis with excision or medical treatment of the neoplasm. Immunohistochemistry may provide evidence of cytokine production (e.g., GM-CSF) by neoplastic cells,[66] as may detection of upregulated cytokine gene expression in neoplastic tissue,[67] although the latter does not localize cytokine production to the neoplastic cells.
   b. Canine neoplasms associated with extreme neutrophilic leukocytosis include rectal adenomatous polyps,[68,69] renal solid and/or tubular carcinomas,[66,70,71] pulmonary neoplasms,[67,72] and metastatic fibrosarcoma.[73]
   c. Feline neoplasms associated with paraneoplastic neutrophilia include mammary carcinoma,[74] squamous cell carcinoma, and dermal adenocarcinoma.
9. LAD type I[57,75]
   a. Type I LAD has been recognized in Irish red and white setters, Irish setters, mixed breed dogs, Holstein cattle, and a domestic long-haired cat; the disorders are referred

to as canine LAD (CLAD), bovine LAD (BLAD), and feline LAD (FLAD). They are caused by mutations in the β2-integrin subunit gene which leads to decreased expression of CD18 (β2-integrin subunit). The defect prevents formation of functional CD11/CD18 heterodimers, which are adhesion molecule receptors needed for firm adherence and transendothelial migration of WBCs.

b. Dogs with CLAD have a persistent neutrophilia beginning as pups and have concurrent myeloid hyperplasia in marrow.[57,76,77] Monocytosis and lymphocytosis may also be present. Affected dogs have an increased susceptibility to infections because WBCs cannot reach sites of infection. Before the specific defect was established, the disorder was called *canine granulocytopathy syndrome*. CLAD dogs can be detected by a PCR assay.[78]

c. Cattle with BLAD have a marked neutrophilia and recurrent infections.[79,80] PCR assays have been used to identify affected cattle and greatly reduce the occurrence of BLAD through selective breeding.

d. The castrated, male domestic longhair cat with FLAD had no expression of CD18 on its WBCs, and it had persistent neutrophilia (> 40,000/μL) exacerbated by recurrent clinical infections; it lived 7 yr.[58]

10. LAD type III:[81,82] LAD III has been reported in a German shepherd dog and a German shepherd–Rottweiler cross with the same mutation in the gene for kindlin-3, thus leading to defective or absent kindlin-3 protein and impaired function of WBC and platelet integrins (adhesion molecule receptors). Kindlin-3 is an intracellular protein necessary for WBC and platelet integrins to undergo conformational changes needed for high-affinity binding and adhesion. The affected dogs had persistent neutrophilia, monocytosis, and lymphocytosis accompanied by recurrent infections and mucosal or postoperative hemorrhage. This mutation can be diagnosed by PCR testing.

11. Other conditions or unknown mechanisms

a. Neutrophilia induced pharmacologically by G-CSF administration

b. Neutrophilia of estrogen toxicosis: Neutrophilia occurs 2–3 wk after estradiol injections.[83]

D. Neutropenia (measured blood neutrophil concentration below the LRL) (Table 2.9)

1. Neutropenia is a decreased concentration of combined neutrophil stages (total neutrophils), not just segmented neutrophils. When the segmented neutrophil concentration is decreased but the band neutrophil is increased, the combined segmented and band neutrophil concentrations may be WRI, in which case neutropenia would not be present. However, similar disorders may be present.

2. Inflammatory neutropenia (Figure 2.9B)

a. Inflammatory neutropenia occurs when margination in vessels or emigration of neutrophils to inflamed tissue exceeds the release of neutrophils from marrow. This typically happens during an overwhelming or severe acute inflammatory disease. If associated with endotoxemia, margination of neutrophils may be a dominant contributor.

(1) Cytokines and chemoattractants stimulate margination and emigration of large numbers of neutrophils to inflamed tissue. This movement may begin within minutes after introduction of an infectious agent. Cytokines (G-CSF, GM-CSF) also stimulate increased production of neutrophils, but the blood changes caused by this stimulation are not expected for at least 2 d (can occur more quickly than in health). The balance of bone marrow chemokines and their receptors is altered

Table 2.9. Disorders and conditions that cause neutropenia
Inflammation
Overwhelming bacterial infections and endotoxemia* (e.g., horses with equine salmonellosis or Potomac horse fever)
Some viral infections causing secondary bacterial sepsis/endotoxemia:* canine and feline parvovirus (myelotoxicity also contributes), equine influenza
Variety of acute inflammatory states in cattle:* mastitis, pneumonia
Mature-neutrophil destruction
Idiopathic IMN
Alloimmune neonatal neutropenia
Hemophagocytic syndrome
Bone marrow lymphocytosis of granular lymphocytes (neoplastic or nonneoplastic)
Granulocytic or generalized marrow hypoplasia (decreased production neutropenia)
Infectious agents:* parvovirus (dogs and cats), FeLV, *Toxoplasma*, *Ehrlichia*
Neoplasms: primary or metastatic
Toxicants (see Chapter 6)
Predictable:* cytotoxic chemotherapeutics, azathioprine, chloramphenicol (cats), endogenous or exogenous estrogen (dogs), trichothecene mycotoxins, bracken fern, colchicine
Idiosyncratic: albendazole, fenbendazole, griseofulvin, phenylbutazone, trimethoprim-sulfadiazine, D-penicillamine
Whole body irradiation
Myelophthisis
Immune-mediated aplastic anemia (including bovine neonatal pancytopenia)
Gelatinous transformation of bone marrow (severe malnutrition)
Anti–G-CSF antibodies caused by recombinant human G-CSF (dogs)
Ineffective production
Precursor-targeted IMN
Phenobarbital-induced cytopenias
Severe cobalamin deficiency
G-CSF deficiency (possibly)
Myelodysplastic neoplasms
Other, unknown, or multiple mechanisms
Trapped-neutrophil syndrome (Border collies)
Toxicants: zonisamide (suspected), lithium carbonate (cats), *Cuscuta capestris* (horses)
Cyclic hematopoiesis
Canine cyclic hematopoiesis (cyclic neutropenia) (mostly grey collies or their crosses)
Cyclic hematopoiesis associated with FeLV
G-CSF administration (acute and transient)
Canine babesiosis

* A relatively common disorder, condition, or mechanism

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Some healthy greyhounds, possibly whippets, and North American Belgian Tervurens have neutrophil concentrations below RIs for most dogs, but Belgian Tervurens in Belgium did not.

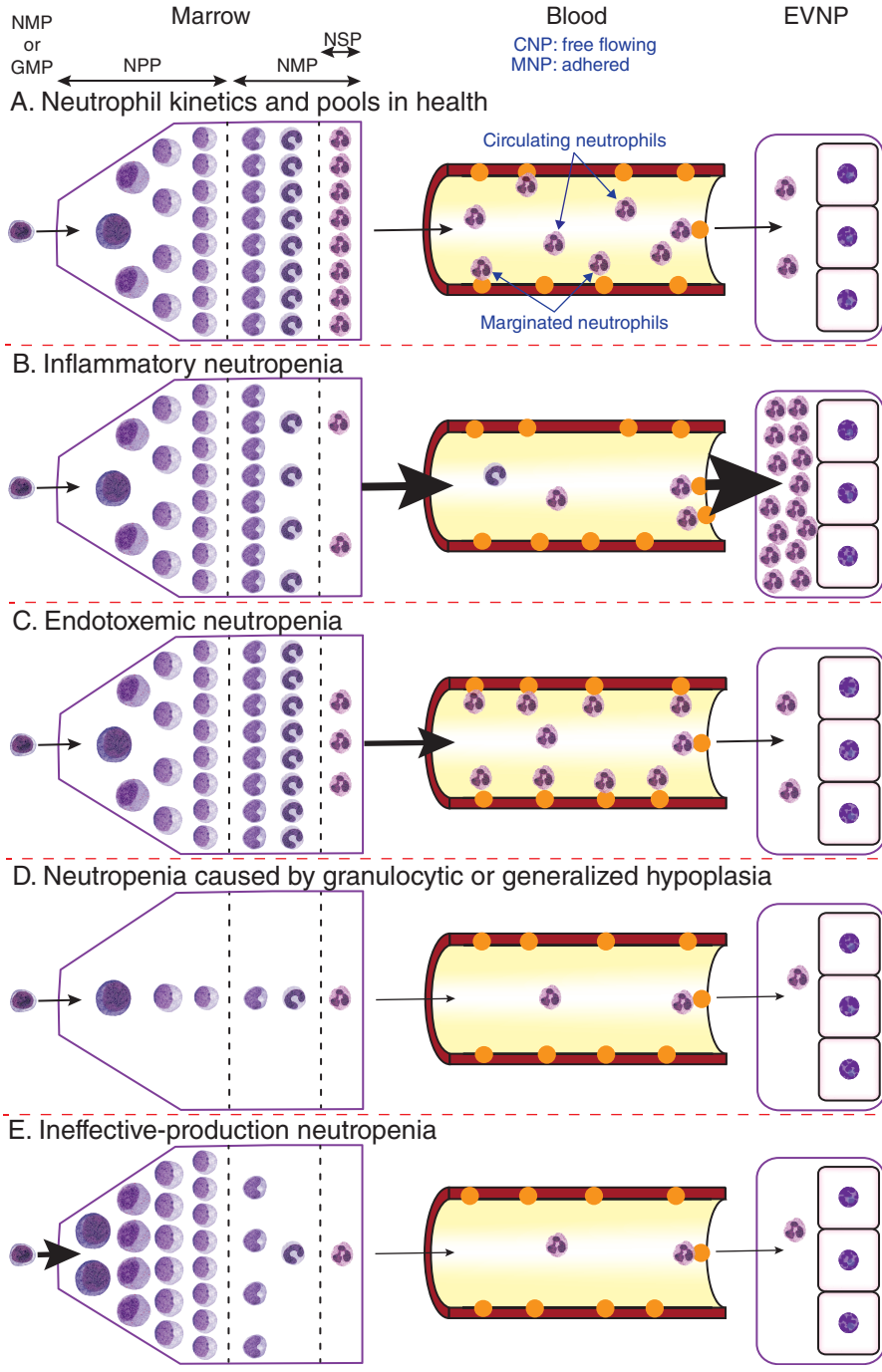


Figure 2.9. Neutropenia kinetics.

A. Neutrophil kinetics in health (a reduced version of Figure 2.2).

B. Inflammatory neutropenia (severe, overwhelming tissue demand): Neutropenia occurs because the margination and migration of neutrophils into the inflamed tissues exceed the release of neutrophils from marrow which has not had time to respond adequately.

by G-CSF or other factors to promote rapid neutrophil egress, mostly from the NSP. With depletion of the NSP, more cells in the NMP are released, and thus a left shift is typically present. However, there may not be a left shift even with the release of cells from the NMP, because the total measured neutrophil concentration may be so low that the band neutrophil concentration cannot be greater than the URL. If bone marrow is examined during the illness, cell populations reflect the changes in the leukon:

(a) Early: depletion of the NSP ± a decreased NMP
(b) 2–3 d: increased NPP without increased NMP
(c) 5–7 d: myeloid hyperplasia; increased NPP and NMP

(2) Inflammatory neutropenia is common in adult cattle because they have a relatively small NSP. When there is a demand for neutrophils, there are not enough neutrophils in the NSP for a sudden release to create a neutrophilia. Also, experimental data suggest that it takes longer for cattle to generate neutrophils once the reserve pool is depleted.[84]

(a) The expected response to an acute inflammatory stimulus in adult cattle is exemplified by the sequence of events after introduction of *Enterobacter aerogenes* into mammary glands of lactating cows.[85]
(i) Initial neutropenia (within 6 h)
(ii) Significant left shift (by 24 h)
(iii) Nadir of neutropenia (by 31 h)
(iv) [Neutrophil] WRI and persistent left shift (by 72 h)

(b) Persistence of a neutropenia past 3 d occurs with more severe infections.
(c) The severity of the neutropenia is typically greater in bacteremic than nonbacteremic cattle with mastitis.[86]

b. Endotoxemic neutropenia: Whenever an infection with Gram-negative organisms causes endotoxemia, endotoxins initiate changes in the leukon that may cause neutropenia (Figure 2.9C).

(1) This neutropenia occurs because endotoxins cause the rapid shift of neutrophils from the CNP to the MNP, a shift mediated by endotoxin-induced release of inflammatory mediators (e.g., TNF and IL-1) that increase expression of various adhesion molecules on neutrophils and endothelial cells. This effect lasts for 1–3 h after a single exposure.

Figure 2.9. *continued*

C. Endotoxemic neutropenia: Neutropenia occurs because endotoxins stimulate the margination of neutrophils (sequestration of neutrophils in the MNP). With the concurrent inflammatory response, a variety of changes in neutrophil kinetics are possible (B and C are often combined). Endotoxins may also cause increased release of neutrophils (see the text).

D. Neutropenia caused by granulocytic or generalized hypoplasia: Neutropenia occurs because neutrophil production is decreased but effective. If infection occurs secondary to the neutropenia, patterns B or C may be superimposed on pattern D.

E. Ineffective-production neutropenia: The pattern varies with the specific disorder, but the commonality is adequate to increased early myelopoiesis without adequate release of neutrophils from the marrow. One cause is marrow-targeted immune-mediated neutropenia, in which the immune system destroys neutrophil precursors, thus causing the appearance of maturation arrest beginning at the targeted stage (shown here at the metamyelocyte stage, but it could occur earlier or later). Cellular atypia is not expected in precursor-targeted immune-mediated neutropenia but may be seen with cobalamin deficiency, phenobarbital toxicity, and a myelodysplastic neoplasm. See the text for other causes of this pattern.

(2) By the time a veterinarian examines an endotoxemic animal, neutropenia may no longer be present because:
  (a) Endotoxins stimulate release of neutrophils from bone marrow in about 8–12 h.
  (b) Endotoxins stimulate increased production of neutrophils, which affects blood neutrophil concentrations in about 3–5 d.
  (c) Other inflammatory mediators may concurrently alter neutrophil kinetics.
  (d) Concurrent endogenous release of epinephrine or cortisol may promote an increased neutrophil concentration.
(3) Most studies of endotoxin-induced changes in neutrophil kinetics involved injection of endotoxins. In spontaneous infections, other factors are probably also altering neutrophil movement.

3. Neutropenia caused by mature-neutrophil destruction
  a. Idiopathic IMN may primarily affect mature neutrophils or their precursors (see precursor-targeted IMN below in Section 5b).[87]
    (1) This condition is seen primarily in dogs. Antineutrophil antibodies are thought to bind to mature neutrophils that are then destroyed by the mononuclear phagocyte system. Destruction may occur in the bone marrow, liver, and spleen; in some affected dogs, phagocytosis of mature neutrophils is apparent in these organs. Causative factors have not been established in domestic mammals, but immunosuppressive therapy typically leads to remission.
    (2) Diagnosis is usually based on exclusion, mild clinical signs compared to those expected with sepsis-induced neutropenia, persistent neutropenia with antimicrobial therapy, consistent bone marrow findings, and apparent response to immunosuppressive therapy. With targeting of mature neutrophils, examination of bone marrow reveals granulocytic hyperplasia, often with more band than segmented neutrophils.
  b. Alloimmune neonatal neutropenia[88]
    (1) Neutropenia occurs when a neonate ingests colostrum and absorbs maternal antineutrophil antibodies formed to paternally derived antigens absent from the dam's neutrophils but present on the neonate's neutrophils. These antibodies bind the neonate's neutrophils and mediate neutrophil destruction.
    (2) Alloimmune neonatal neutropenia has been reported in foals; the diagnosis has been supported by exclusion of other causes of persistent neutropenia and by neutrophil agglutination or antibody immunofluorescence assays that used controls but that lacked formal validation or extensive use with equine samples.
  c. Neutropenia of hemophagocytic syndrome:[89–93] Neutropenia may be one of multiple cytopenias associated with nonselective phagocytosis of hemic cells by nonneoplastic, cytokine-stimulated macrophages. Acquired hemophagocytic syndrome may occur secondary to a variety of inflammatory (including immune-mediated) or neoplastic diseases. Although anemia and thrombocytopenia are common in dogs with hemophagocytic histiocytic sarcoma, a disorder with similarities to hemophagocytic syndrome, neutropenia is not.[94]
  d. Neutropenia associated with bone marrow lymphocytosis of granular lymphocytes (neoplastic or nonneoplastic): Activated granular lymphocytes (NK or T cells) express increased Fas ligand, which may bind to its receptor (Fas) on neutrophils, thus inducing apoptosis and premature neutrophil destruction. This may occur in some cases of idiopathic IMN, and the mechanism has been proposed to contribute to

neutropenia occurring several weeks after feline immunodeficiency virus infections,[95] and also to neutropenia associated with granular cell leukemias in people. In dogs, neutropenia has rarely been associated with T-cell granular lymphocytic leukemia or lymphoma involving the bone marrow but with no lymphocytosis in blood.[96,97]

4. Neutropenia caused by granulocytic or generalized marrow hypoplasia (decreased-production neutropenia) (Figure 2.9D)
   a. Granulocytic hypoplasia or aplasia may occur when stem cells or cells of the marrow microenvironment are damaged (see Chapter 6). There may be generalized or more selective hypoplasia causing decreased neutrophil production. It may occur with infections, toxicants (including endogenous estrogens), whole body irradiation, myelophthisis, immune-mediated disease including bovine alloimmune neonatal pancytopenia, severe malnutrition with gelatinous transformation of bone marrow, or decreased G-CSF caused by chronic treatment with recombinant human G-CSF, which can induce antibodies that cross react with a dog's own G-CSF. When caused by cytotoxic chemotherapeutic agents that damage neutrophil precursors in addition to dividing neoplastic cells, the [neutrophil] nadir typically occurs 7–10 d after administration. Vincristine can also cause neutropenia at the low doses used to treat immune-mediated thrombocytopenia; concurrent cyclosporine therapy increases the risk.[98]
   b. This cause of neutropenia is usually differentiated from other causes of neutropenia by persistence of neutropenia (often without a left shift, though secondary infections may cause left shifts and toxic change) and bone marrow examination findings. Typically, maturation within the neutrophilic cell line is complete if neutropoiesis is just reduced and not absent. Because the disease may damage other cell lines in the marrow, concentrations of other blood cells (e.g., RBCs or platelets) may be decreased. There may not be a lymphopenia, because lymphopoiesis in other lymphoid tissues may not be impaired and blood [lymphocyte] is very dependent on lymphocyte recirculation.
5. Ineffective-production neutropenia (neutropenia of ineffective neutropoiesis) (Figure 2.9E)
   a. Ineffective production occurs in several conditions in which neutrophil precursors die before release from marrow. It is characterized by persistent neutropenia (with little or no left shift unless sepsis develops secondary to neutropenia) concurrent with a myeloid maturation arrest (a marked decrease of cells beyond a certain maturation stage) or prominent myeloid hyperplasia if the problem is affecting the latest maturation stages, including their migration from the marrow. A monocytosis may be present because GM-CSF and G-CSF increase the number of cells differentiating toward monocytes. Lymphocyte concentrations typically are WRI.
   b. Precursor-targeted IMN:[99] Some dogs with glucocorticoid-responsive neutropenia and a clinical diagnosis of IMN have early- to mid-stage myeloid maturation arrest without the prominent neutrophilic hyperplasia seen with immune-mediated destruction of mature neutrophils (Section 3a above). This parallels findings in some dogs with precursor-targeted immune-mediated anemia. Primary precursor-targeted IMN was suspected in a cat with otherwise unexplained, persistent neutropenia that appeared to have a lasting response to immunotherapy (and concurrent antibiotics).[100]
   c. Phenobarbital-induced cytopenias: Clinically significant isolated neutropenia, bicytopenia, or often pancytopenia occur in up to 5 % of dogs receiving phenobarbital for seizures, typically after a few months of treatment.[101,102]

Neutropenia is associated with myeloid hyperplasia (ineffective neutropoiesis), often with a left shift and increased marrow phagocytosis of myeloid cells, and it most commonly resolves within 2–3 wk of discontinuing phenobarbital and switching to a different anticonvulsant. Similar changes may be seen in the erythroid lineage, and dysplastic cells may be present in some cases. The mechanism of cytopenias is not known, but immune-mediated cell destruction may contribute.

d. Severe cobalamin deficiency: Rare mutations in genes for amnionless or cubilin, the two proteins forming intestinal (and renal) cubam receptors, cause intestinal malabsorption of cobalamin, failure to thrive, and ineffective or decreased hematopoiesis with dysmyelopoiesis and dyserythropoiesis.[103–106] Such mutations have been documented in several canine breeds, including giant schnauzers, Australian shepherds, Border collies, beagles, and komondors. The dogs respond quickly to parenteral cyanocobalamin therapy.

e. Chronic idiopathic neutropenia was reported in a Rottweiler that had undetectable G-CSF deficiency by a bioassay.[107] The dog's marrow had granulocyte cell populations indicative of a maturation arrest. Investigators suggested this was because G-CSF is needed for terminal neutrophil differentiation, but no G-CSF treatment was attempted. Immunosuppressive therapy was given for only 4 d, not long enough to exclude precursor-targeted IMN.

f. Myelodysplastic neoplasms involving neutropoiesis are typically characterized by cytopenia with marrow expansion of precursors in the cytopenic lineage. Differentiating these neoplasms from hyperplastic responses may be difficult, but prominent cellular atypia (dysplastic change) would support a myelodysplastic neoplasm.

6. Other, unknown, or multiple mechanisms

a. Trapped-neutrophil syndrome, an inherited autosomal recessive condition similar to human Cohen syndrome, occurs in Border collies with neutropenia or low-normal [neutrophil], microcephaly, narrow and elongated skull, developmental delay, and secondary infections.[108,109] Glucocorticoid-responsive neutrophilic polyarthritis (thought to be immune mediated) has been reported in some affected dogs, and other inflammatory lesions indicate that a large number of neutrophils can reach at least some sites of inflammation despite persistent neutropenia.[109,110] Affected dogs have a mutation in the vacuolar protein sorting 13B gene (*VPS13B*) involved in glycosylation, sorting, and transport of proteins, but the mechanism by which the mutation causes neutropenia is not known. Affected dogs have myeloid hyperplasia with increased neutrophil death and some evidence of neutrophil phagocytosis.[109,111] PCR testing may be used to confirm the mutation.

b. Toxicities

(1) Neutropenia was attributed to idiosyncratic reactions to zonisamide therapy in four dogs with otherwise unexplained neutropenia that resolved within days to 6 mo after discontinuation of the anticonvulsant.[112] Bone marrow was analyzed in only one of the dogs and was supportive of ineffective myeloid hyperplasia rather than hypoplasia; this dog was also receiving phenobarbital, which is known to cause neutropenia and ineffective myeloid hyperplasia, but neutropenia eventually resolved without cessation of phenobarbital.

(2) Lithium carbonate administration to cats caused neutropenia and what was described as a maturation arrest with decreased mature neutrophils in the bone marrow.[113] It was not clear from the report if early myeloid stages were expanded

(which would support ineffective neutropoiesis) or if there was just depletion of the storage pool.

(3) Alfalfa dodder, caused by ingestion of the rootless herb *Cuscuta campestris*, has caused neutropenia in horses, but the cause is not known and might be secondary to colitis and sepsis.[114]

c. Cyclic hematopoiesis

(1) Canine cyclic hematopoiesis (cyclic neutropenia) of grey collies, grey collie crosses, and other dogs[115,116]

(a) Canine cyclic hematopoiesis is a rare autosomal recessive disorder in grey collies and grey collie crosses, and it has been reported in a mixed breed dog without a collie phenotype. Its diagnostic features are caused by approximately 2-wk cycles of neutrophil, RBC, platelet, and monocyte production resulting in cyclic neutropenia. The cycle of neutrophil production is out of synchrony with the other cycles.

(b) Affected dogs are susceptible to infections because of recurrent neutropenia and usually grow ill prior to 6 mo of age. Anemia is usually mild because the life span of RBCs is longer than the production cycles. Thrombocytosis may be present, and defective platelet function has been described.

(c) It is caused by a mutation of *AP3B1*, the gene encoding the β-subunit of *adaptor-related protein complex 3* (AP3), a protein involved in intracellular trafficking of vesicular proteins.[117] Neutrophils of affected dogs have decreased elastase protein in primary granules, decreased elastase activity, and accumulation of an elastase precursor protein outside of their primary granules during maturation, supporting a defect in protein trafficking between organelles.[118] Additionally, impaired protein trafficking in melanocytes and platelets causes hypopigmentation and defective platelet function. Human cyclic neutropenia is caused by mutations in the gene (*ELANE*) encoding neutrophil elastase, and other cases of human congenital neutropenia involve mutations in either *ELANE* or *AP3B1*. The precise pathogeneses of canine cyclic hematopoiesis and cyclic neutropenia remain speculative.[116,119]

(2) Cyclic hematopoiesis associated with FeLV infection[120]

d. G-CSF administration causes a rapid and transient decrease in [neutrophil] that may cause neutropenia 30 min after administration with return to baseline by 60 min.[121] G-CSF binds to neutrophil receptors and causes transient activation of CD18, thus mediating rapid neutrophil margination, largely in lungs and spleen.

e. Neutropenia of canine babesiosis: Neutropenia, often with a left shift and sometimes with toxic change, accompanies hemolytic anemia and commonly thrombocytopenia in at least a moderate percentage of dogs with *Babesia canis* (especially *B. canis canis*) infections.[122,123] The left shift and toxic change are supportive of inflammation, but the cause of neutropenia may be multifactorial. Notably, the disease is not associated with neutrophilia, a common finding in dogs with immune-mediated hemolytic anemia.

7. The [WBC], [neutrophil], and [lymphocyte] of greyhounds and healthy North American Belgian Tervurens may be lower than those of other breeds,[124,125] and findings for [WBC] may be similarly low in whippets.[126] In contrast, these values in Belgian Tervurens in Belgium were comparable to other breeds, although the [WBC] decreased with age.[127]

II. Abnormal lymphocyte concentrations
  A. Lymphocytosis (increased measured blood lymphocyte concentration; the term lymphocytosis is also used to describe an increase in lymphocytes in other tissues or wash fluids) (Table 2.10)
    1. Chronic inflammatory lymphocytosis (Figure 2.10B)
      a. Lymphocytosis may occur with subacute or chronic inflammation, but it is most often absent in chronic inflammatory conditions. When present, it is caused by increased lymphopoiesis in response to chronic antigenic or cytokine stimulation. Reactive lymphocytes may be present. Causes include infections by certain bacteria (e.g., *Ehrlichia canis*), fungi, parasites, or viruses (e.g., FeLV, BLV), and immune-mediated disease (e.g., immune-mediated hemolytic anemia in cats). Substantial nonneoplastic lymphocytosis occurs sporadically with a variety of infectious and noninfectious inflammatory disorders in cats.[128]
      b. In chronic inflammatory disorders, lymphocytosis is part of a hyperplastic lymphoid system. There may be concurrent enlarged lymph nodes or lymphoid hyperplasia in other tissues.
      c. Lymphocytosis is usually mild to moderate (2–3× URL), but occasionally marked (> 30.0×10^3/μL in dogs, cats, or cattle). When moderate or marked, immunophenotyping and PARR may be used to assess for evidence of lymphocytic leukemia.
      d. Concurrent leukogram abnormalities commonly include neutrophilia (mature, perhaps with right shift or left shift) and/or monocytosis and occasionally eosinophilia and/or basophilia.

Table 2.10. Disorders and conditions that cause lymphocytosis

Chronic inflammation
Bacterial infections, especially anaplasmal* (e.g., *Ehrlichia canis*)
Fungal infections, primarily systemic
Viral infections: FeLV, BLV, equine infectious anemia virus
Protozoal infections, especially *Babesia* and *Theileria*
Immune-mediated hemolytic anemia (cats)
Increased catecholamines (physiologic lymphocytosis)
Fight-or-flight response:* excitement, fright, pain, exercise, anxiety
Catecholamine injections: epinephrine or norepinephrine
Lymphoproliferative neoplasms
Lymphoma (BLV, FeLV, and idiopathic), leukemic phase*
Lymphoid leukemia
BLV infection causing persistent lymphocytosis of cattle*
Idiopathic polyclonal B-cell lymphocytosis of English bulldogs (mostly)
Hypoadrenocorticism (Addison's disease)
Thymoma (nonneoplastic T-cell lymphocytosis)
Leukocyte adhesion deficiency, types I and III

* A relatively common disorder, condition, or mechanism

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Young post-neonatal domestic mammals typically have higher lymphocyte concentrations than mature animals of the respective species. Bovine RIs that include data from cattle infected with BLV mask detection of lymphocytosis because BLV-infected cattle tend to have greater lymphocyte concentrations, even when apparently healthy.

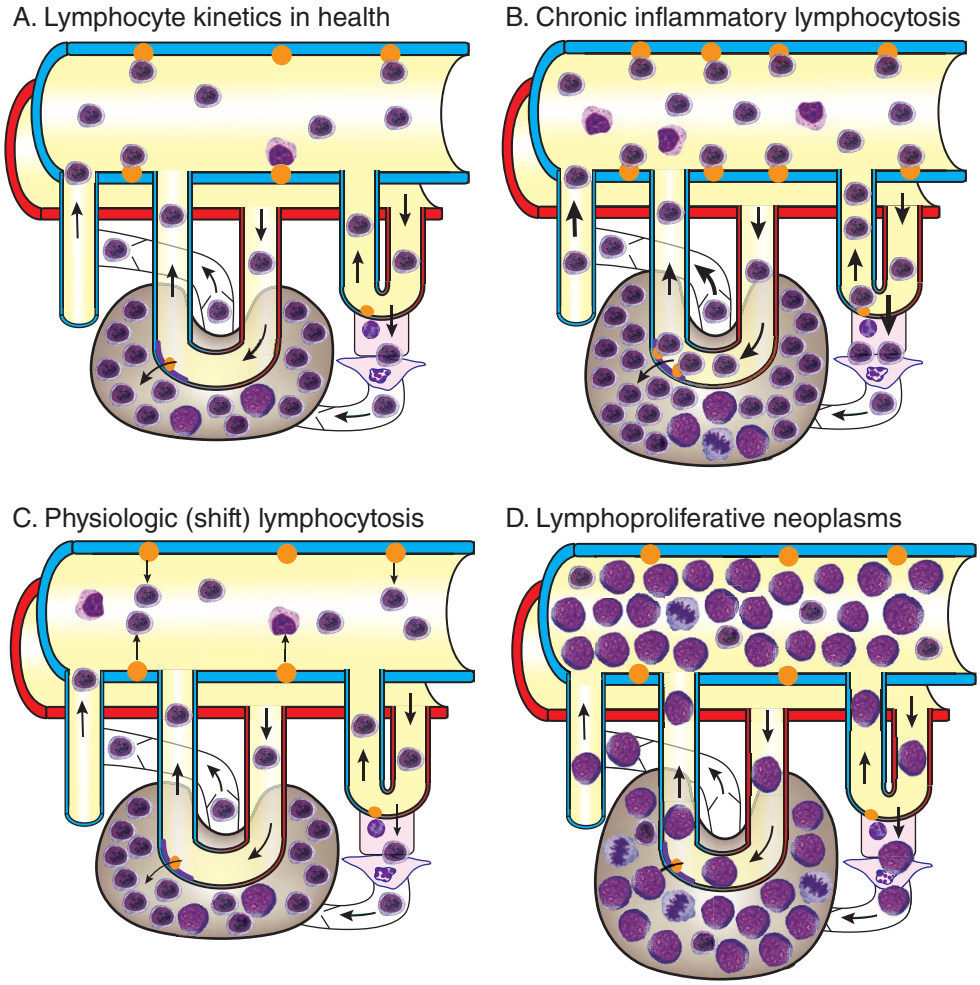


Figure 2.10. Lymphocytosis kinetics.

A. Lymphocyte kinetics in health. Blood lymphocytes are distributed between marginal and circulating pools. Certain subpopulations migrate into lymph nodes via high endothelial venules where they interact with other cells. Most of the entering lymphocytes and any new lymphocytes generated in the node leave through efferent lymphatics that empty into blood at the thoracic duct. From blood, lymphocytes enter other tissues via specific adhesion molecule interactions with endothelial cells (except spleen, which they enter through red pulp). Lymphocytes that enter nonlymphoid tissue may remain or enter the afferent lymphatic vessels and be transported to regional lymph nodes. This lymphocyte recirculation promotes contact of each T and B lymphocyte with the specific antigen each is able to detect (in lymph nodes or other sites), and it provides a route for expanded clones of activated lymphocytes to reach tissues once an antigen is detected.

B. Chronic inflammatory lymphocytosis. Lymphocytosis occurs because of increased lymphopoiesis associated with the immune response to an inflammatory agent. Granular lymphocytes may be prominent, e.g., in some dogs infected by *Ehrlichia canis*. The lymphocytosis is a part of lymphoid hyperplasia.

C. Physiologic (shift) lymphocytosis: Lymphocytosis occurs because of the shift of lymphocytes from the MLP to the CLP. Granular lymphocytes may be noticeably but mildly increased.

D. Lymphoproliferative neoplasms: Acute lymphoblastic leukemia has infiltrated lymph nodes and other tissues, or the leukemic phase of a lymphoma has led to infiltration of bone marrow and blood. In either case, lymphocytosis results from an uncontrolled proliferation of a lymphocyte clone. Note: Lymphocytosis usually does not accompany lymphoma, whereas lymphopenia is common because of impaired lymphocyte recirculation (not shown).

2. Increased catecholamines causing a physiologic (shift) lymphocytosis (Figure 2.10C)
   a. This lymphocytosis is caused by shifting of lymphocytes from the MLP to the CLP (especially from the spleen) that is promoted by exercise or exogenous or endogenous catecholamines. The lymphocyte shift is probably mediated through increased blood flow rate, decreased lymphocyte adherence to endothelium, and splenic contraction.[65,129]
   b. The magnitude of lymphocytosis may be up to 2× URL, and the lymphocytosis usually lasts minutes to hours.
   c. Morphologic changes in lymphocytes are not expected other than potentially an increase in granular lymphocytes. This is because catecholamines appear to preferentially demarginate cytotoxic T and NK lymphocytes by binding to their high-affinity β_2-adrenoreceptors and reducing their adhesion to endothelial cells.[65,129] An increase in NK cell concentration has been reported in calves subjected to transport stress.[130]
   d. As with shift neutrophilia, shift lymphocytosis is seen most frequently in cats, young horses, and healthy animals.
3. Lymphoproliferative neoplasms (Table 6.5 and Figure 2.10D)
   a. This lymphocytosis usually is caused by a neoplastic proliferation of lymphoid cells in lymph nodes, bone marrow, or other tissues.
   b. BLV and FeLV are known to induce neoplastic transformation of lymphocytes, but not all lymphoid neoplasms in cats and cattle are caused by these viruses. Causes of lymphoid neoplasms in other domestic mammals are not known.
   c. In domestic mammals, lymphoid leukemias may originate from the spleen or bone marrow, or they may represent the leukemic manifestations of lymphoma, i.e., the neoplasm originates in the lymph nodes or other lymphoid tissues outside the bone marrow and spreads to the bone marrow and blood.
   d. Leukemia is often not recognized until there is a marked or extreme lymphocytosis, but the increases begin as mild and progress. In some lymphoid leukemias, lymphocytes have abnormal or "immature" morphologic features (acute leukemias) while in others they appear individually similar to cells seen in health but are too numerous or monotypic (chronic lymphocytic leukemias). The diagnosis of leukemia is easiest when cells are atypical and numerous; however, special testing may be needed to confirm a lymphocyte lineage. When the lymphocytes lack definite atypia, a diagnosis of chronic lymphocytic leukemia may require exclusion of other disorders and evidence of clonality.
4. BLV infection causing persistent lymphocytosis of cattle: BLV infections may induce a mild to marked persistent polyclonal B-cell lymphocytosis; the pathogenesis appears to involve decreased apoptosis (increased survival) of virus-infected B lymphocytes combined with altered T lymphocyte function and proliferation of noninfected B lymphocytes.[131] A subset of cattle with BLV-induced persistent lymphocytosis develops clonal lymphocyte proliferations (neoplasms). Sheep also develop lymphocytosis from BLV infection, but it may be more progressive because of different effects of the virus.
5. Idiopathic polyclonal B-cell lymphocytosis of English bulldogs (mostly): This syndrome is characterized by mild to marked lymphocytosis (median = 22,000/μL) of small to intermediate B lymphocytes lacking overt atypia and commonly associated with polyclonal or oligoclonal IgA ± IgM hyperglobulinemia.[132] Routine and extended PARR results have not supported clonality. Splenomegaly or splenic masses are commonly present, and bone

marrow lymphocytosis (20–40 % lymphocytes) was present in the two cases for which bone marrow findings were reported. Although evidence for a neoplastic proliferation is lacking in these dogs, lymphocytes may undergo malignant transformation. Rarely, a similar constellation of findings has been seen in dogs of other breeds.

6. Lymphocytosis of hypoadrenocorticism (Addison's disease)
   a. This lymphocytosis may be caused by an absence of glucocorticoid hormones, which normally inhibit lymphocyte production or alter lymphocyte distribution in the body.
   b. Although dogs with hypoadrenocorticism may have a lymphocytosis, many do not. Always think of hypoadrenocorticism if an obviously stressed dog has a neutropenia and lymphocytosis (stress should cause the opposite, i.e., neutrophilia and lymphopenia), especially in azotemic dogs and when the [eosinophil] is near the URL or increased.
   c. The classic leukogram of canine hypoadrenocorticism consists of a low-normal to decreased neutrophil concentration, high-normal to increased lymphocyte concentration, a monocyte concentration WRI, and a high-normal to increased eosinophil concentration. However, variations occur, and mild neutrophilia may be present.
7. Thymoma-associated T-cell lymphocytosis has been reported in dogs.[133–135] Lymphocytosis is polyclonal and resolves with thymectomy (when thymectomy is possible). The lymphocytes lack overt atypia and may accumulate in blood because of dysregulated production (overproduction), increased escape from the thymus, or altered trafficking.
8. LAD: Lymphocytosis accompanies neutrophilia and monocytosis in some dogs with type I and type III LAD (see neutrophilia section).
9. Post-neonatal young animals may appear to have lymphocytosis if results are compared to adult RIs.
   a. Although lymphocyte concentrations over the first few months of life are generally low compared to those at maturity, values typically peak at a few months of age and then decline. Therefore, for example, a 3-mo-old kitten or juvenile cria could appear to have a lymphocytosis if adult RIs are used for comparison.[136,137] In dogs, the [lymphocyte] decreases by about 800/μL over the first 2–4 yr of life;[138] it may continue to decrease mildly with age.[139] Foal lymphocyte concentrations generally increased over the first 3 mo of life to reach values approximating common URLs for adults;[140,141] mild decreases in [lymphocyte] appear to occur with age.[142] Lymphocyte concentrations in cattle increase until about 1 yr of age, and then gradually decrease over the years in adults.[55]
   b. Compared to adult RIs, the apparent lymphocytosis may be up to 2× URL.
10. Bovine hematologic RIs have widely incorporated data from cattle infected with BLV. Consequently, URLs for lymphocyte concentrations are generally too high for noninfected, healthy cattle, and lymphocytosis is under-recognized.[131]

B. Lymphopenia (measured blood lymphocyte concentration below the LRL) (Table 2.11)
   1. Acute inflammatory lymphopenia (Figure 2.11A)
      a. Contributors to acute inflammatory lymphopenia may include decreased lymphopoiesis and the following changes in lymphocyte kinetics stimulated by acute inflammatory mediators:
         (1) Increased margination and emigration of lymphocytes to inflamed tissue
         (2) Homing of lymphocytes to lymph nodes by increasing the rate of migration through postcapillary high endothelial cells

Table 2.11. Disorders and conditions that cause lymphopenia
Acute inflammation
Acute bacterial infections ± endotoxemia*
Acute viral infections (see text for examples)*
Increased glucocorticoids causing steroid ("stress") lymphopenia*
"Stress" (physical or neurogenic)*
Hyperadrenocorticism
Glucocorticoid therapy*
Adrenocorticotropic hormone administration
Depletion
Lymphoid effusion: chylothorax and feline cardiomyopathy (promoted by repeated removal)
Loss of lymph: alimentary lymphoma, enteric neoplasms, granulomatous enteritis, paratuberculosis, protein-losing enteropathy, lymphangiectasia, ulcerative enteritis
Destruction of lymphocytes: cytotoxic drugs, whole body irradiation, certain viruses
Congenital lymphoid hypoplasia or aplasia
Severe combined immunodeficiency (SCID) of horses (Arabian, Appaloosa, and Caspian) and dogs (basset hound, Cardigan Welsh corgi, Jack Russell terrier, and Frisian water dog)
Common variable immunodeficiency (horses)
Anemia/immunodeficiency syndrome of Fell and Dales ponies (Fell Pony syndrome)
Multicentric lymphoma (destruction of lymphoid tissue)

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Some healthy greyhounds and North American Belgian Tervurens have lymphocyte concentrations below RIs for most dogs, whereas Belgian Tervurens in Belgium did not.

(3) Reducing the rate of lymphocytes leaving lymph nodes via efferent lymphatic vessels
(4) Lymphocyte apoptosis associated with changes in cytokine concentrations, particularly in sepsis or severe disease

b. Most acute inflammatory leukograms with neutrophilia or neutropenia also have lymphopenia. Disappearance of lymphopenia is generally considered a good prognostic sign.
c. Commonly, lymphopenia is considered to be caused by the stress associated with acute inflammation rather than caused by the inflammatory process itself. The stress of an illness may or may not induce or contribute to the lymphopenia.
d. Lymphocyte depletion plays a role in the acute inflammatory lymphopenia caused by certain viruses, e.g., bovine viral diarrhea virus, canine distemper virus, parvovirus, corona viruses, feline immunodeficiency virus, feline panleukopenia virus, feline infectious peritonitis virus, and severe fever with thrombocytopenia syndrome virus.

2. Increased glucocorticoids causing steroid ("stress") lymphopenia (Figure 2.11B): Typical causes are the same as mentioned for steroid neutrophilia. Lymphopenia is caused by the changes in lymphocyte kinetics mediated by endogenous or exogenous glucocorticoids. Severity and duration of lymphopenia are generally proportional to dose and duration of increased glucocorticoids.

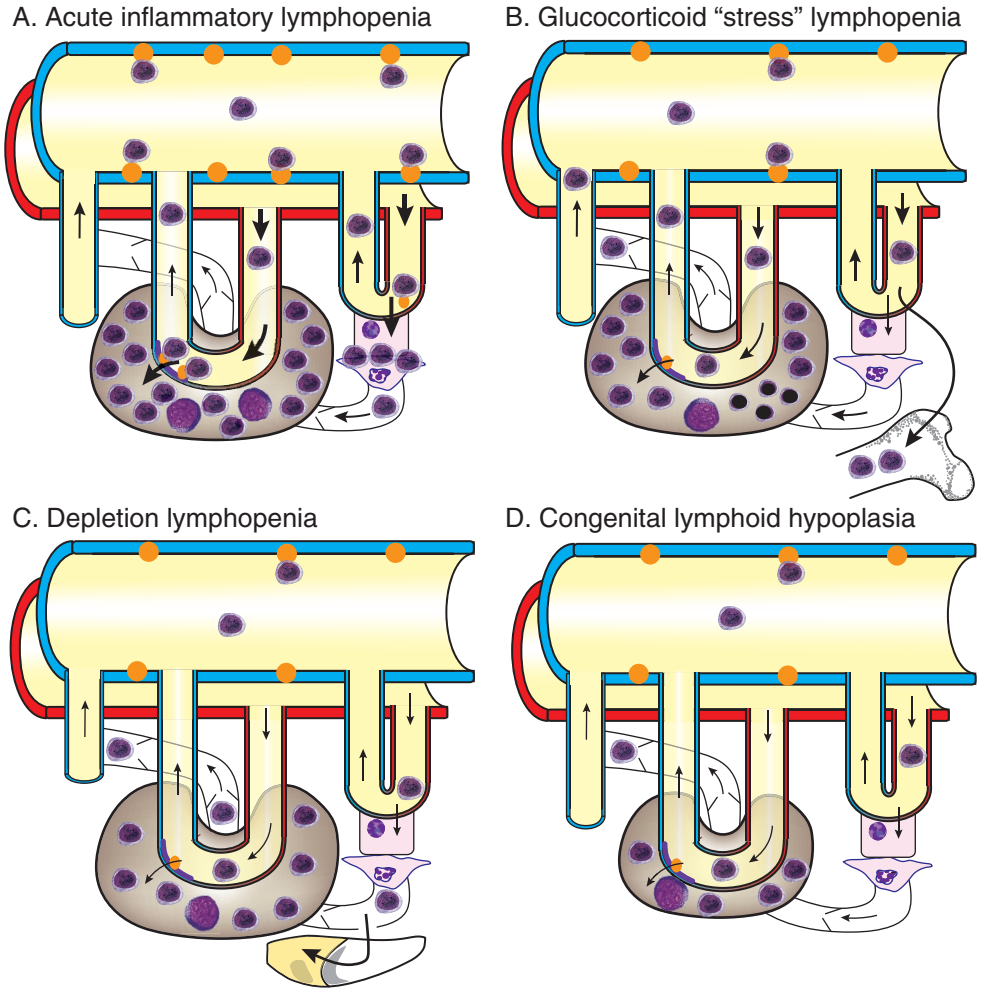


Figure 2.11. Lymphopenia kinetics.

A. Acute inflammatory lymphopenia: Lymphopenia occurs because of (i) increased margination and migration of lymphocytes to inflamed tissue, (ii) homing of lymphocytes to lymphoid tissues, and (iii) decreased movement of lymphocytes from lymph nodes back to blood.

B. Glucocorticoid ("stress") lymphopenia: Soon after administration of glucocorticoids, lymphopenia occurs because of the movement of lymphocytes to lymphoid tissues (including marrow) and decreased efflux of lymphocytes from lymph nodes. With persistent administration, glucocorticoids can become lymphotoxic and thus destroy lymphocytes in lymph nodes and other tissues (note pyknotic cells with condensed nuclei).

C. Depletion lymphopenia: Lymphopenia occurs because lymphocytes are lost from the vascular system with a loss of lymph or lymph-rich fluid (e.g., lymphangiectasia, repeated drainage of a chylous effusion; pleural effusion shown) or when there is lymphoid depletion in lymphoid organs (e.g., cytotoxic drugs, lymphotoxic viruses).

D. Congenital lymphoid hypoplasia: Lymphopenia occurs because of decreased lymphocyte production. It is more severe when the defect impairs T lymphocyte production because T lymphocytes are the predominant type of blood lymphocyte in health. When congenital lymphoid aplasia is present, lymphocytes are rare and lymphoid tissue is scant.

a. Lymphocytes shift from the CLP to other pools. Once the effects of the glucocorticoids diminish, the lymphocytes return to blood. Some reports indicate the lymphopenia is caused by decreased efflux of lymphocytes from lymph nodes,[143] whereas other data indicate a redistribution to bone marrow.[144,145]
b. Lymphotoxic effects may cause lymphoid hypoplasia and thus decreased lymphopoiesis, particularly at greater glucocorticoid concentrations. Lymphocytes subtypes vary in their sensitivity to glucocorticoid-induced apoptosis,[146] and there are likely species differences. The degree to which apoptosis contributes to steroid-induced lymphopenia in domestic mammals is not clear.

3. Depletion lymphopenia (Figure 2.11C): This lymphopenia is produced by a loss of lymphocytes from the body or by their destruction in the body.
a. Loss of lymphocyte-rich lymph can occur with lymphangiectasia or because of an incomplete lymphocyte circulation pathway (e.g., repeated removal of chylothoracic fluid from a cat). Disorders that cause this lymphopenia are not common.
b. Lymphocytes may be destroyed by cytotoxic drugs, irradiation, or certain viruses (e.g., bovine viral diarrhea virus, canine distemper and parvovirus viruses, feline immunodeficiency virus), thus depleting lymphocytes from certain tissues and blood.

4. Lymphopenia of congenital lymphoid hypoplasia or aplasia (Figure 2.11D): This lymphopenia is caused by congenital lymphoid hypoplasia or aplasia that decreases lymphocyte production and promotes infections. Lymphopenia may be mild to marked. Because most blood lymphocytes are T lymphocytes, selective aplasia of T lymphocytes causes a more severe lymphopenia than selective aplasia of B lymphocytes.
a. Moderate or severe lymphopenia occurs in horses and dogs with different forms of congenital severe combined immunodeficiency (SCID).[147–150]
b. Persistent or intermittent lymphopenia with severe B cell lymphopenia occurs in horses with common variable immunodeficiency.[151]
c. Mild lymphopenia with decreased B lymphocytes develops by 3 wk after birth in some Fell and Dales pony foals with anemia/immunodeficiency syndrome (Fell pony syndrome); their lymphocyte concentrations do not increase with age as expected.[152,153] Other features of the syndrome are severe anemia, thymic hypoplasia, absence of plasma cells, failure to produce IgM and probably IgG (colostral IgG is present), opportunistic infections, and death by about 3 mo.

5. Lymphopenia of multicentric lymphoma: Lymphopenia is common in animals with lymphoma. The lymphopenia may be caused by decreased production (damage to lymph nodes) or altered lymphocyte kinetics (disrupted circulation patterns).
6. The [WBC], [lymphocyte], and [neutrophil] of healthy greyhounds[125] and Belgian Tervurens in North America[124] but not Belgium[127] may be lower than those of other breeds.

III. Abnormal monocyte concentrations
A. Monocytosis (increased measured blood monocyte concentration) (Table 2.12)
1. Inflammatory monocytosis: Acute and chronic infectious or noninfectious inflammatory diseases may cause monocytosis by cytokine stimulation of monocyte production and release. It appears that monocyte maturation and release from the bone marrow can be markedly accelerated by inflammation, with newly formed monocytes entering the blood within several hours after endotoxin administration.[21] Although

Table 2.12. Disorders and conditions that cause monocytosis
Inflammation (acute or chronic)
Infections:* bacterial (including anaplasmal and rickettsial), fungal, protozoal
Necrosis:* hemolysis, hemorrhage, burns, neoplasms, infarcts, trauma, sterile inflammation
Neoplasms (via generation of inflammatory mediators in addition to necrosis)
Sterile foreign body/material
Increased glucocorticoids
"Stress" (physical or neurogenic)*
Hyperadrenocorticism
Glucocorticoid therapy*
Adrenocorticotropic hormone administration
Monocytic leukemia (rare)
IMN
Cyclic hematopoiesis
Leukocyte adhesion deficiency, types I and III
G-CSF administration

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

commonly stated otherwise, monocytosis does not indicate that inflammation is chronic. Monocytosis generally reflects a need for macrophages in diseased tissue or blood (e.g., necrosis, hemorrhage, granulomatous inflammation).

2. Increased glucocorticoids causing a steroid ("stress") monocytosis: Glucocorticoid hormones or drugs are a common cause of monocytosis in dogs and may contribute to mild monocytosis in cats;[154] they cause minimal to no changes in horses and cattle. Causes are the same as those for a steroid neutrophilia or lymphopenia. Monocytosis is probably caused by a shift in monocytes from a marginated pool to the circulating pool.
3. Monocytic leukemia: This is typically characterized by a marked monocytosis when diagnosed. Monocytes may appear abnormal or relatively normal, but when atypical and immature, they would probably not be categorized as monocytes.
4. Monocytosis secondary to IMN: Because neutrophils and monocytes share a common bipotential stem cell, there may be increased monocytopoiesis from increased cytokine stimulation when there is ineffective neutropoiesis.
5. Cyclic hematopoiesis: Mild monocytosis may occur during neutropenic cycles and herald increases in blood neutrophil concentrations.
6. LAD: Monocytosis accompanies neutrophilia and lymphocytosis in some dogs with type I and type III LAD (see neutrophilia section).
7. G-CSF: This may be administered to promote neutropoiesis, but it also promotes monocytopoiesis.[155]

B. Monocytopenia: This is difficult to document because healthy domestic mammals may have relatively few blood monocytes; reported LRLs are often 0/μL and up to 200/μL across species. Monocytopenia is not considered a diagnostic problem. However, endotoxemia may rapidly decrease blood [monocyte] or cause monocytopenia as monocytes rapidly marginate or migrate to inflammatory sites.[21]

IV. Abnormal eosinophil concentrations
  A. Eosinophilia (increased measured blood eosinophil concentration; the term eosinophilia is also used to describe an increase in eosinophils in other tissues or wash fluids)
    1. Eosinophilia may be transient (present on a single CBC) or persistent (present on repeated CBCs) and associated with many different organs and disorders, often with disorders involving the lungs, skin, or gastrointestinal tract.[156] The stimulus for eosinophilia may not be clear, especially for transient eosinophilias, but many persistent eosinophilias are caused by allergic disease, extravascular tissue-dwelling parasites, infections, or neoplasms (Table 2.13). In one study, ~20 % of dogs with eosinophil concentrations ≥ 1500/μL had neoplasms, often of lymphocytes or mast cells, but other factors might have been involved.[157] (Note: Although marked eosinophilia was considered to be ≥ 1500/μL in that study, some laboratory URLs for canine [eosinophil] are of that magnitude or greater.)

Table 2.13. Disorders and conditions that cause eosinophilia

Hypersensitivity (allergic) disorders
Flea-bite dermatitis*
Hypersensitivity to staphylococcal or streptococcal proteins
Milk allergy in ruminants
Asthma and eosinophilic respiratory disorders
Parasitism
Ectoparasites
Tissue nematodes, trematodes, and protozoa*
Dogs: *Dirofilaria*, *Angiostrongylus*, *Acanthocheilonema* (*Dipetalonema*), *Spirocerca*, *Strongyloides*, *Trichuris*, *Paragonimus*, *Filaroides*, *Crenosoma*, *Eucoleus*, and *Oslerus*; larval migration of hookworms and roundworms; *Habronema*
Cats: *Paragonimus*, *Eucoleus*, *Oslerus*, and *Aeleurostrongylus*
Horses: *Strongyloides*
Cattle: *Sarcocystis*
Pythiosis (gastrointestinal or cutaneous/subcutaneous)
Mast cell degranulation caused by inflammation:* cutaneous, respiratory, intestinal, genital, urinary
Hypoadrenocorticism
Idiopathic eosinophilic conditions
Dog:* eosinophilic myositis, eosinophilic gastroenteritis, eosinophilic panosteitis, eosinophilic pneumonitis, eosinophilic granuloma complex in Siberian huskies
Cat:* eosinophilic granuloma complex, eosinophilic enteritis, hypereosinophilic syndrome, gastrointestinal eosinophilic sclerosing fibroplasia
Idiopathic hypereosinophilic syndrome
Paraneoplastic eosinophilia (including mast cell neoplasms)
Chronic eosinophilic leukemia
Other myeloid or lymphoid neoplasms

* A relatively common disorder, condition, or mechanism

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Eosinophilia may be missed in greyhounds, whippets, or other dogs with nonstaining or myeloperoxidase-deficient eosinophils.

a. With hypersensitivity disorders, typically there are clinical signs associated with the involved tissues, e.g., pruritus with flea bite or staphylococcal dermatitis, respiratory problems with allergic bronchitis.
b. When eosinophilia is caused by a parasitic infection, it is likely a reaction to a parasite (adult or larva) in extravascular tissues and not in the lumen of the intestines. Ectoparasites (e.g., fleas) or endoparasites may cause eosinophilia. Endoparasites are most commonly filarids, nematodes, and trematodes, but some protozoan infections such as in sarcocystosis and toxoplasmosis may also cause eosinophilia. Most organisms that infect blood cells (e.g., hemotropic *Mycoplasma*, *Babesia*, and *Cytauxzoon*) are not expected to cause eosinophilia, although eosinophilia was reportedly the most common hematologic abnormality in one study of dogs infected with *Hepatozoon canis* (data and RIs were not reported).[158]
c. *Pythium insidiosum*, a hypha-forming fungus-like oomycete, infects mammals (e.g., dogs, horses), and causes gastrointestinal or cutaneous/subcutaneous pythiosis. Eosinophilia is common.
d. Eosinophilia has been associated with inflammation in a variety of mast cell-rich tissues, although establishing a direct causal relationship may be difficult.
e. Eosinophilia, or more often a high-normal eosinophil concentration, is found in some dogs with hypoadrenocorticism. In one study, eosinophilia occurred in a greater percentage of Great Pyrenees dogs (38 %) than dogs of other breeds (14 %) with hypoadrenocorticism.[159] The pathogenesis probably involves diminished cortisol.
f. Several idiopathic eosinophilic conditions associated with eosinophilia typically cause clinical signs associated with the involved tissues (Table 2.13).
g. Idiopathic hypereosinophilic syndrome is occasionally found in cats, dogs, and horses.[160–163] Rottweilers appear to be predisposed.[164,165] The syndrome is characterized by persistent and often marked eosinophilia of undetermined cause (reactive or neoplastic) associated with eosinophilic infiltration of multiple organs, often causing organ dysfunction. Clinical progression may exclude a neoplastic process.

2. Paraneoplastic eosinophilia
   a. Mast cell neoplasms and adjacent tissues may contain eosinophils because of chemoattractants released from mast cells. This occurs most commonly in dogs. There may be concurrent eosinophilia, rarely marked, which may be caused by factors released from neoplastic cells.[166]
   b. Eosinophilia can occur with other neoplasms that might release eosinophilia-inducing factors (e.g., IL-5). Paraneoplastic eosinophilias have been reported in dogs, cats, and horses with the following neoplasms.[167]
      (1) Dogs: T-cell lymphoma, B-cell lymphoma (likely),[157,168] thymoma, mammary carcinoma, oral fibrosarcoma, and rectal adenomatous polyp
      (2) Cats: transitional cell carcinoma, T-cell lymphoma, and alimentary lymphoma
      (3) Horses: intestinal lymphoma
3. Chronic eosinophilic leukemia (Table 6.5): This form of myeloproliferative neoplasm is very uncommon. In cats, it has been linked to a retroviral infection.[169] Definitive differentiation from nonneoplastic eosinophilia is usually not possible, particularly at first presentation. Findings that increase the likelihood of leukemia include increased blood or bone marrow myeloblasts, dysplastic changes in eosinophils or other hemic cells, cytopenias, and persistence of eosinophilia.
4. Other eosinophilia-associated myeloid or lymphoid neoplasms include other myeloproliferative neoplasms, myelodysplastic/myeloproliferative neoplasms, acute myeloid leukemias, and acute lymphoid leukemias.

Table 2.14. Disorders and conditions that cause eosinopenia
Increased glucocorticoids*
Stress (physical or neurogenic)*
Hyperadrenocorticism
Glucocorticoid therapy*
Adrenocorticotropic hormone administration
Inflammation*
Diseases causing a hypoplastic to aplastic marrow

* A relatively common disorder, condition, or mechanism

Note: As for other low-concentration WBC types, decreased results may not be reliable because of the imprecision of WBC differential counts. Also, RIs for eosinophil concentrations in many species have 0/μL as the LRL, so eosinopenia cannot be detected; when that is the case, the listed disorders and conditions may result in low-normal values.

5. Eosinophilia may be missed with either automated or microscopic WBC differentials in individuals with abnormal eosinophil granules (see Nonstaining eosinophil granules (grey eosinophils) of dogs).

B. Eosinopenia (measured blood eosinophil concentration below the LRL)
   1. By itself, eosinopenia is of little diagnostic significance. However, eosinopenia may be part of an inflammatory or glucocorticoid leukogram or occur with decreased production from generalized bone marrow disease (Table 2.14).
   2. Because eosinophil concentrations in healthy animals are typically low, and the calculated eosinophil concentration of a CBC may not be accurate, eosinopenia in CBC results may not truly represent a pathologic state. Also, RIs for eosinophil concentrations in many species have 0/μL as the LRL, so eosinopenia cannot be detected.

V. Abnormal basophil concentrations
   A. Hematology analyzers do not reliably identify basophils; increases may be missed when present or they may be reported when not present. Also, canine and feline basophils are commonly misidentified by microscopy.
   B. Basophilia (increased measured blood basophil concentration, but the term is also used to mean increased basophils in other tissue or fluids, or, in another context, staining by basic dyes)
      1. Only substantial increases or persistent mild increases in basophil concentrations above 200–300/μL should be considered definitive basophilias, because of the imprecision of WBC differential counts, especially when dealing with a minority cell population. However, because so few basophils are present in health, undetected increases in [basophil] may be present without a reported basophilia.
      2. A cause of basophilia may not be apparent. Basophilia is associated with allergic, parasitic, and neoplastic (including paraneoplastic) states (Table 2.15), and often accompanied by eosinophilia.[170]
   C. Basopenia (decreased blood basophil concentration): This cannot be documented with routine WBC differential counts because basophil concentrations are typically very low in domestic mammals (the LRL is 0/μL). Basopenia is not known to be clinically significant.

Table 2.15. Disorders and conditions that cause basophilia
Allergic reactions (immediate or delayed)
Drugs, foods, inhalants, and insect stings or bites
Parasitism
Fleas
Gastrointestinal parasites such as nematodes
Vascular parasites:* *Dirofilaria immitis* and *Acanthocheilonema (Dipetalonema) reconditum*
Neoplasms (including paraneoplastic basophilia)
Basophilic leukemia
Mast cell neoplasm
Lymphoma, particularly T-cell lymphoma
Thymoma (dogs)
Lymphomatoid granulomatosis
Myeloproliferative neoplasms (chronic myeloid leukemia, essential thrombocythemia, polycythemia vera)

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

VI. Abnormal mast cell concentrations
  A. Mastocytemia (mast cells detected in systemic blood)
    1. Finding one mast cell in a blood film or buffy coat preparation of domestic mammals is considered to indicate the presence of mastocytemia. The term *mastocytemia* is preferred over *mastocytosis* because mastocytosis may refer to increased mast cell numbers in tissues other than blood. Either term may refer to neoplastic or nonneoplastic mast cell populations.
    2. Disorders that may cause mastocytemia (Table 2.16)
      a. Cutaneous and subcutaneous mastocytomas, which are common neoplasms of dogs, may spread to blood and hemic organs.[171] So, too, may mucosal and visceral forms.
      b. Several nonneoplastic mastocytemia disorders have been reported in dogs (Table 2.16). Most such disorders are associated with acute inflammatory diseases.[172–175]
      c. Mast cell leukemias are rare; when present, mast cells are numerous in bone marrow and blood but may also infiltrate other tissues.
      d. Mastocytemia is rarely reported in cats. When present, it is usually associated with visceral mast cell neoplasms, but it has been reported in cats with no evidence of mast cell neoplasms and with diagnoses of lymphoma or metastatic hemangiosarcoma.[176,177]
    3. Some articles that describe mastocytemic animals report the number of mast cells found per blood film. This *number per blood film* should be considered descriptive and not quantitative because the amount of blood examined is not constant or is unknown.
  B. Because no mast cells are expected in the blood of healthy domestic mammals, decreased concentrations do not occur.

Table 2.16. Disorders reported to be associated with mastocytemia
Neoplastic disorders
Cutaneous mast cell neoplasms
Visceral mast cell neoplasia
Mast cell leukemia
Other neoplasms in cats (lymphoma, hemangiosarcoma)
Nonneoplastic disorders in dogs
Inflammatory*
Enteritis, especially parvovirus
Fibrinous pericarditis and pleuritis
Bacterial peritonitis
Aspiration pneumonia
Acute pancreatic necrosis
Immune-mediated hemolytic anemia
Renal failure associated with acute inflammation
Skin diseases: flea-bite hypersensitivity, atopy, sarcoptic mange, and food allergy; some with secondary pyoderma
Hemorrhage secondary to hemophilia in dogs
Gastric volvulus in dogs

* A relatively common disorder, condition, or mechanism

LEUKOGRAM PATTERNS

I. Alterations in blood WBC concentrations frequently occur in patterns that are summarized in Table 2.17. Recognizing a leukogram pattern aids in the understanding of an animal's illness. Recognizing glucocorticoid and physiologic leukocytosis patterns reduces overinterpretation of data as indicative of inflammatory disease. However, not all disorders produce classic patterns, concurrent processes can complicate patterns, and some patterns overlap considerably. All leukogram results may even be WRIs when an animal has an inflammatory disease or even an infectious disease. The characteristic features of most patterns are seen in all domestic mammals, but there are significant species differences.
   A. Dogs
      1. Typically, dogs have the most pronounced acute inflammatory leukocytosis.
      2. The classic glucocorticoid (steroid or "stress") pattern is probably most common in dogs.
   B. Cats
      1. Higher MNP:CNP ratios[7] plus their fight-or-flight responses make cats more prone to a physiologic leukocytosis.
      2. FeLV infections can create alterations in cell concentrations and appearance.
   C. Horses
      1. Horses can mount an inflammatory neutrophilia, but many equine inflammatory diseases produce neutropenia. Equine neutrophils are typically more segmented and have more irregular nuclear margins than those of other species. With inflammation, a significant number of their circulating neutrophils may not meet some criteria for band neutrophils (smooth, parallel nuclear margins) and yet they may have decreased segmentation and broader, paler nuclei than mature neutrophils. These cells are sometimes classified as *hyposegmented neutrophils* and contribute to evidence of a left shift and inflammation.
      2. A pronounced left shift is uncommon without toxic neutrophils.

Table 2.17. Interpretation of major leukogram patterns (see the text for species differences)

Total WBCs	Segmented neutrophils	Nonsegmented neutrophils	Lymphocytes	Monocytes	Eosinophils	Interpretation
↑	↑	↑	↓	WRI–↑	↓–WRI	Inflammation, likely acute; ± concurrent glucocorticoid effect
↑	↑	WRI–↑	WRI–↑	WRI–↑	WRI–↑	Inflammation, acute or chronic
↑↑	↑↑	WRI–mild ↑	WRI–↑	WRI–↑	WRI–↑	Inflammation, chronic
↑	↑	WRI–mild ↑	↓	↑	↓	Glucocorticoid leukocytosis or inflammation
↑	↑	WRI	↑	WRI–↑	WRI	Physiologic leukocytosis or inflammation
↓	↓	WRI–↑	↓	WRI	↓–WRI	Acute overwhelming inflammation ± endotoxemia[a]
↓–WRI	↓	WRI	WRI	WRI–↑	WRI	Decreased or ineffective neutropoiesis[b]
WRI–↑	↓–↑	WRI	WRI–↑	WRI	WRI–↑	Consider hypoadrenocorticism; may reflect inflammation
↑↑↑	↓–WRI	WRI	↑↑↑	↓–WRI	WRI	Lymphoid leukemia, acute or chronic[c]

[a] This pattern could occur with sepsis secondary to decreased neutrophil production.

[b] There may be a left shift and secondary inflammation if neutropenia resulted in sepsis. If decreased neutrophil production is caused by myelophthisis from leukemia, the [WBC] may be increased.

[c] With acute myeloid leukemia, atypical cells might be reported as lymphocytes when they are actually myeloid cells with a lymphoid appearance, but they likely would be reported as atypical or blast cells and thus not increase reported concentrations of any specific WBC type; concentrations of nonneoplastic lineages might be decreased.

D. Cattle
   1. Common acute inflammatory states in adult cattle (such as mastitis or pneumonia) cause inflammatory neutropenia because of the relatively small neutrophil storage pool, but neutrophilia may occur in calves with acute inflammation and in adults with chronic suppurative diseases.
   2. Inflammatory states can cause marked lymphocyte atypia that is very difficult to differentiate from neoplastic changes.
   3. BLV infections alter blood lymphocyte concentrations and may cause persistent lymphocytosis or lymphocytic leukemia.
   4. For several decades after the early 1960s, widely used RIs for cattle indicated that the lymphocyte concentration was typically greater than the neutrophil concentration. This was associated with use of a guideline that a neutrophil concentration to lymphocyte concentration ratio > 1 was supportive of inflammation in cattle. However, more recent data from healthy cattle with negative test results for BLV indicate that the mean ratio of neutrophil concentration to lymphocyte concentration is mildly > 1.[131,178]

E. Alpacas and llamas tend to have greater eosinophil concentrations than other species (even with good anthelmintic control), and their baseline neutrophil concentrations are typically greater than those of ruminants. Abnormal WBC patterns are generally similar to other species.

II. Hemic neoplasms (see Chapter 6 for more information)
   A. Leukogram patterns in animals with hemic neoplasms are extremely variable. When doing a differential WBC count, if a nucleated cell population (not just a cell or two) does not fit clearly into one of the WBC groups seen in health, the cells should be categorized as "other" or, if appropriate, "blast" cells rather than force them into a classification that is inappropriate. Evaluation by a veterinary clinical pathologist is indicated.
      1. Extreme leukocytosis due to numerous poorly differentiated cells is indicative of an acute leukemia or the leukemic phase of lymphoma. Microscopic findings may suggest that the poorly differentiated cells are of a particular lineage (WBC, erythroid, megakaryocytic, or lymphoid), but the cells often cannot be classified without phenotyping. Concurrent neutropenia and thrombocytopenia are common.
      2. Extreme leukocytosis due to numerous well-differentiated cells is suggestive of chronic leukemia, but a marked inflammatory or paraneoplastic leukogram must be excluded.
      3. Low numbers of atypical cells in blood, particularly if accompanied by cytopenias, suggest the need to examine bone marrow, lymph nodes, or spleen for the source of the atypical cells. Hemic neoplasia may be prominent in the bone marrow, lymph nodes, or spleen when it is not obvious in the blood.
      4. Marked, unexplained, inappropriate rubricytosis is an indication for bone marrow examination to evaluate for a hemic neoplasm of erythroid origin (typically in cats).
      5. Hemic neoplasms may be present without alterations in CBC results.
   B. Light-scatter patterns produced by some hematology analyzers (e.g., the CELL-DYN 3500) may provide clues as to the type of neoplastic cells that are present. This is because neoplastic cells often have similar light-scatter properties as their nonneoplastic counterparts.[179] However, such findings are typically not definitive.
   C. Multiple methods are used to characterize neoplastic hemic cells in an attempt to establish their lineage. Other than the microscopic examination of routinely stained blood, marrow, or lymph node samples, several other methods are used to evaluate cells (see Chapter 6).
      1. Enzyme cytochemistry or histochemistry
      2. Immunocytochemistry or immunohistochemistry

3. Immunophenotyping via flow cytometry
4. Antigen receptor gene rearrangement assays to detect clonal cell populations

D. Not all atypical cell populations in blood are of hematopoietic origin. Rarely, enough disseminating atypical nucleated cells are present to suspect *carcinocythemia*, the presence of carcinoma cells in the blood. This has been reported in dogs and cats with advanced, disseminated mammary carcinomas or carcinomas of unknown origin.[180–182]

ABNORMAL MORPHOLOGIC FEATURES OF WBCs

I. Changes associated with inflammatory diseases
   A. *Toxic neutrophils* do not indicate toxemia or exposure to a toxicant as the name may suggest but are instead neutrophils with maturation defects caused by rapid neutropoiesis from inflammation or G-CSF administration. They can be particularly useful to recognize when leukogram changes are not otherwise clearly indicative of inflammation; this includes when the [WBC] and differential findings are WRI or with neutrophilias and patterns that could reflect either inflammation or responses to glucocorticoids or catecholamines. They can appear in blood within 3 h after an inflammatory stimulus.[183]
      1. The following so-called *toxic changes* that define toxic neutrophils (Figure 2.12) arise in the bone marrow or a site of extramedullary hematopoiesis.

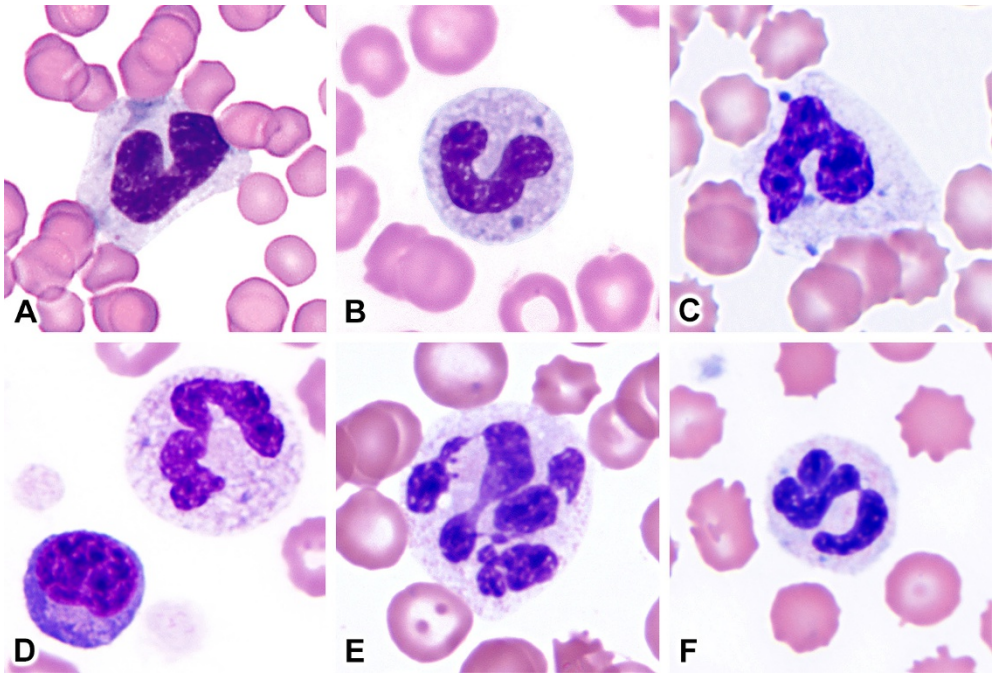


Figure 2.12. Toxic neutrophils (A–E) and a pseudo-toxic neutrophil (F). A. Toxic band neutrophil with large irregular Döhle body (*top*), mild diffuse basophilia, and mildly foamy cytoplasm, horse. B–D. Band neutrophils (B and C) and segmented neutrophils (D) with Döhle bodies, cytoplasmic foaminess, and mild to moderate diffuse cytoplasmic basophilia, dogs (also a reactive lymphocyte in D). E. Toxic giant segmented neutrophil with cytoplasmic basophilia, dog. F. Punctate Döhle-like bodies in a segmented neutrophil, 15-h-old blood that had lacked toxic change when fresh, dog.

a. *Foamy cytoplasm* is cytoplasmic clearing due to dispersed organelles. It is often referred to as vacuolation, but it should not be confused with the discrete vacuoles that develop in neutrophils exposed to EDTA for a few hours.[184]
b. *Diffuse cytoplasmic basophilia* is retention or persistence of cytoplasmic RNA during maturation. If cytoplasmic basophilia is in only nonsegmented neutrophils, it may reflect the immaturity of the cell and not be a toxic change.
c. *Cytoplasmic Döhle bodies* (focal cytoplasmic basophilia) are irregular, round to angular, blue-grey cytoplasmic structures that are aggregates of rough endoplasmic reticulum that contain RNA.
d. *Asynchronous nuclear maturation* is characterized by nuclei that have finely granular, pale, broad lobes (suggesting immaturity) separated by filaments or pronounced indentations (suggesting maturity). Criteria for classifying neutrophils as band or segmented forms are based on synchronous maturation; the criteria are difficult to apply to toxic neutrophils.
e. *Giant neutrophils* are larger segmented or band neutrophils that are released from marrow because of asynchronous cellular maturation, likely because of skipped mitoses in the NPP. Some appear to contain twice the normal amount of nuclear material, sometimes with two distinct nuclei. They are seen most often in cats. They appeared in the blood of horses 36 h after endotoxin administration, and many were reported at 48–72 h.[33] If not accompanied by other toxic changes, giant neutrophils may be caused by poodle marrow dyscrasia, FeLV infections in cats, cobalamin deficiency, chemotherapy with certain drugs, or as part of a neoplastic proliferation of neutrophils.
f. Hyalinized nuclei have been considered a toxic change but may also represent deterioration or autolysis.
g. *Toxic granules* in human neutrophils are enzyme-containing (e.g., elastase, myeloperoxidase) and acid mucopolysaccharide-containing lysosome-like granules (primary granules) formed in neutrophil precursors (the progranulocyte stage) that remain prominent in later stages instead of becoming inconspicuous as they do in health.[185] They stain more basophilically than secondary granules, and they tend to be unevenly distributed. They are uncommon in domestic mammals and seen mostly in horses. If all of an individual's neutrophils have unusually prominent and diffusely spread magenta cytoplasmic granules, particularly without other toxic changes, they may represent staining or individual variation rather than toxic change. This is most common in foals.

2. Clinically healthy cats may have small Döhle bodies in up to approximately 50 % of the neutrophils, with < 1/3 of the affected neutrophils containing more than two Döhle bodies (unpublished data from Dr. Julia Stickle based on blood smears from over 40 healthy cats). If such numbers of Döhle bodies are detected or reported, they should not be interpreted as indicative of inflammation. The presence of these "toxic changes" in neutrophils of healthy cats can explain why WBC and neutrophil concentrations were WRI in about 50 % of cats with toxic neutrophils, and in 43 % of them, toxic change was the only hematologic suggestion of inflammation (no left shift and WBC and neutrophil concentrations WRI).[186]
3. Neutrophils with toxic changes are commonly associated with severe bacterial infections but can be found in noninfectious states,[187] including after G-CSF administration, in turpentine-induced inflammation,[188] and in dyscrasias induced by cefonicid or cefazedone.[189] Just the presence of a left shift is not a toxic change.

4. Toxic changes can be graded based on types and degrees of toxic changes, but classification systems have not been standardized and there is a lack of data regarding the progression of toxic changes in response to various degrees of inflammation (i.e., which changes occur with which degrees of inflammation).
    a. In general, the more numerous and prominent the toxic changes are, the more severe the inflammatory state is likely to be. Severe toxic change has been associated with poorer prognosis in dogs,[187] and moderate to severe toxic change was associated with poorer outcomes in horses.[190]
    b. Grading systems used to reach these conclusions have been relatively complex, requiring the differentiation of what % of neutrophils is affected (< 10 %, 10–30 %, > 30 %) by focal basophilia (Döhle bodies), diffuse basophilia, "vacuolation" (cytoplasmic clearing), and nuclear hypersegmentation in addition to the degree (mild, moderate, or marked) of each cytoplasmic change.[187,190] The reproducibility of such grading among individuals and laboratories is likely only moderate,[191] and it has not been shown to alter patient management or outcome compared to simply reporting the presence of toxic change and noting when it appears to be severe, an approach used by the author's (MAS) laboratory for many years.
5. Toxic neutrophils (especially band and metamyelocyte stages) can be confused with monocytes when they become diffusely basophilic, particularly if they are large.
    a. If such a cell is a toxic band, similar toxic changes should be present in segmented neutrophils.
    b. In some cases, immature toxic neutrophils cannot be reliably distinguished from monocytes on a Romanowsky-stained blood film. In these cases, accuracy of a WBC differential count may not be as important as recognizing that left shifts and toxic changes indicate that animals have severe inflammatory diseases.
6. Döhle-like bodies can form in vitro over time, more so at room temperature than at 4 °C.[192] These are often single small punctate basophilic inclusions rather than the larger and more varied Döhle bodies seen with inflammation (Figure 2.12F). Their consistent appearance in few to many neutrophils of an aged sample suggests an in vitro phenomenon rather than in vivo toxic change, although other degenerative changes might be present, further mimicking toxic change. This storage lesion is one of many reasons that smears of fresh rather than old blood should be evaluated.

B. Hypersegmented neutrophils
1. *Hypersegmented neutrophils* have increased numbers of definitive nuclear lobes separated by filaments, five or more in most species and perhaps seven or more in horses.[56]
2. They most commonly represent aged neutrophils that remain longer in circulation because of decreased apoptosis or downregulated adhesion molecules caused by increased blood concentrations of glucocorticoids (see Abnormal WBC Concentrations in Blood, Section I.B). The aging effect can also be seen as an in vitro artifact of delayed sample analysis, although most such neutrophils develop swollen nuclei and appear more immature.
3. Neutrophil hypersegmentation also occurs from noninflammatory causes (see Nuclear hypersegmentation unrelated to cell aging).

C. Pseudo-Pelger–Huët neutrophils and eosinophils
1. Pseudo-Pelger–Huët neutrophils are seen in cows[193] and occasionally in horses, alpacas, llamas, and dogs with inflammatory disease. The transient defect represents asynchronous neutrophil maturation with hyposegmented neutrophils but mature chromatin; some neutrophils look like Pelger–Huët cells (see Pelger–Huët anomaly in

the following Section V), but others do not. Usually, other toxic changes are also present with inflammation.

2. Pseudo-Pelger–Huët eosinophils have been described in cattle and a horse.[163,193] In cattle, they were found concurrent with pseudo-Pelger–Huët neutrophils. In the horse, they were found concurrent with an extreme eosinophilia.
3. Pseudo-Pelger–Huët cells also form in certain hemic neoplasms and from exposure to certain drugs and ionizing radiation (see Noninflammatory pseudo-Pelger–Huët anomaly in the following Section VI).

D. Reactive lymphocytes (Figure 2.13)

1. *Reactive lymphocytes* (plasmacytoid lymphocytes, immunocytes, transformed lymphocytes, and virocytes) represent either stimulated T lymphocytes or B lymphocytes and can be found in the blood of animals with a variety of acute and chronic inflammatory diseases. They are common in young animals that are constantly being exposed to new environmental antigens.
2. Reactive lymphocytes can be found in most blood samples, but increases are nonspecific evidence of inflammatory disease. Recognition of increases is subjective and may be affected by lymphocyte appearance, lymphocyte concentration, and the percentage of lymphocytes that appear reactive.
3. Reactive lymphocytes may have a variety of structural features that are considered to be reactive changes, the most consistent being an increased amount of cytoplasm and increased cytoplasmic basophilia (blueness). Other features that may be present are a perinuclear halo, a prominent focal Golgi zone, and eccentric, enlarged, cleft, convoluted, bilobed, or mitotic nuclei; some cells may appear plasmacytoid. A relatively large amount of cytoplasm without basophilia is not clear evidence of reactive change, because some granular lymphocytes (large granular lymphocytes) typically have more cytoplasm than

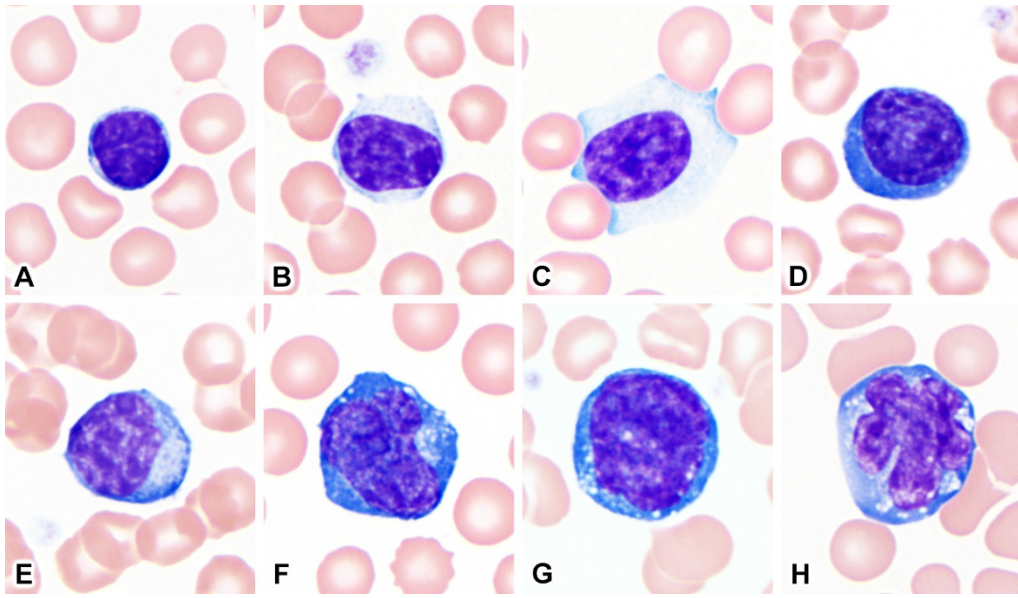


Figure 2.13. Reactive lymphocytes (D–H) in contrast to unreactive small (A), intermediate (B), and large (C) lymphocytes, dog.

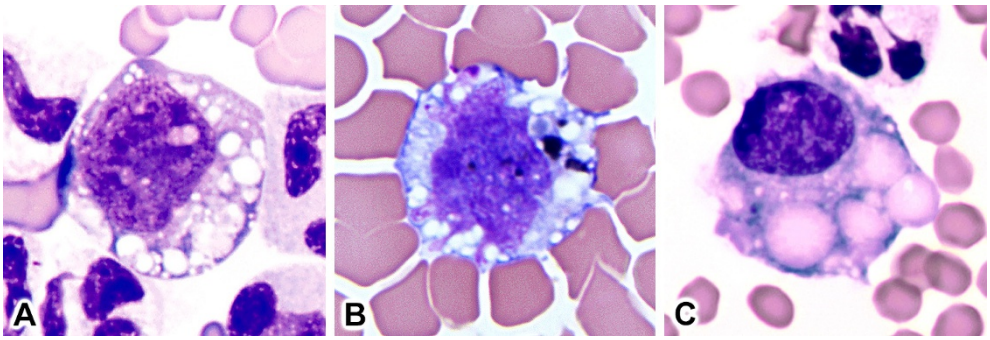


Figure 2.14. Activated monocytes or macrophages in blood. A. Feathered edge, cat with histoplasmosis (no organisms shown). B. Dark inclusions suggestive of hemosiderin, dog. C. Erythrophage, foal with neonatal isoerythrolysis.

other lymphocytes. However, an increased concentration of granular lymphocytes may indicate a reactive (inflammatory) process.

4. Individual reactive lymphocytes can be impossible to distinguish from neoplastic lymphoid cells, especially in cattle. They may also be difficult to differentiate from immature or reactive monocytes and perhaps early to middle stages of nRBCs.

E. Changes in monocytes associated with inflammation: Monocytes can have increased cytoplasmic basophilia and round to ovoid (more immature-shaped) nuclei (*reactive monocytes*) that may make them indistinguishable from reactive lymphocytes, but either cell type is evidence of inflammation. They may also appear macrophage-transformed with large round to oval nuclei and relatively abundant blue-grey cytoplasm with or without vacuoles (Figure 2.14). Such cells are seen in some inflammatory diseases, including systemic fungal diseases, ehrlichiosis, leishmaniasis, and immune-mediated disorders. In one retrospective study, the rare presence of at least three macrophage-like cells in a blood smear occurred in dogs with severe inflammation or neoplasia and was associated with neutropenia, a left shift, thrombocytopenia, anemia, bicytopenia, and pancytopenia.[194] In some cases, the macrophage-like cells were likely neoplastic histiocytes. Although inflammatory macrophages may develop from circulating monocytes, they also might enter blood from other tissues (e.g., liver, spleen, bone marrow).

II. Infectious agents in blood WBCs

A. Bacteria other than those in the family *Anaplasmataceae* (rare) (Figure 2.15A–C)

1. Using a routine Romanowsky stain, basophilic bacterial rods or cocci may be found within neutrophils of bacteremic patients, sometimes accompanied by extracellular bacteria. These patients are often terminal. When these bacteria are present, one must attempt to determine whether the bacteria represent bacteremia or a sample contaminated with bacteria (check sample handling).
2. Mycobacterial bacilli may be present in blood smears from patients with systemic *Mycobacterium* spp. infections. They are unique in that they do not stain with a Romanowsky stain (negative staining reaction) and thus appear as one or often more clear bacterial rods within macrophages or neutrophils.

B. Bacteria in the family *Anaplasmataceae* (order *Rickettsiales*): morulae of *Ehrlichia* spp. and *Anaplasma* spp. (Figure 2.15D–F)

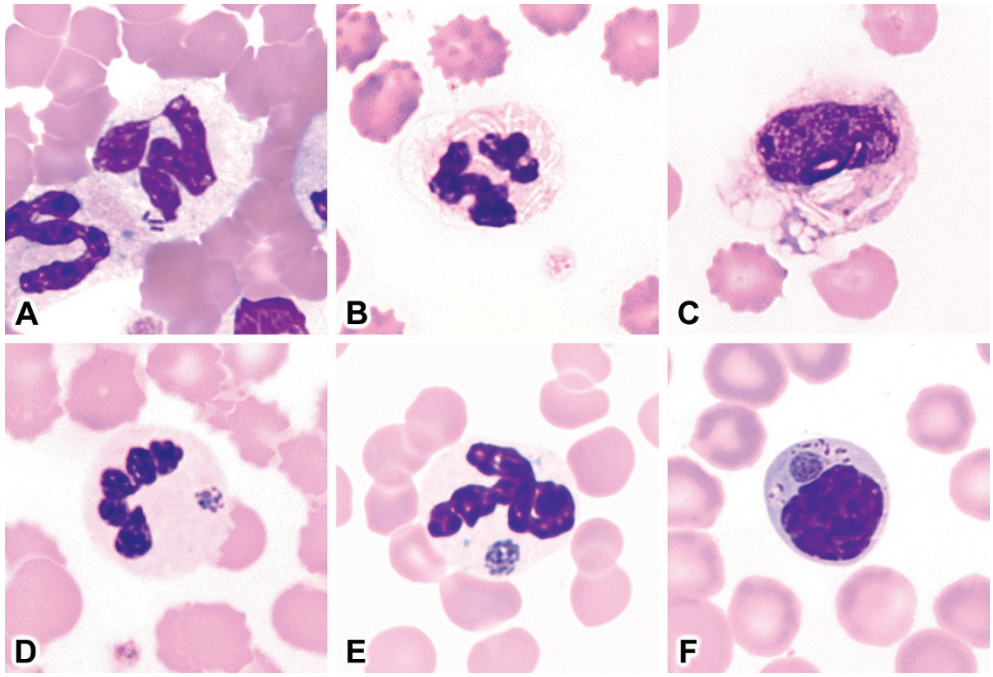


Figure 2.15. Bacteria in WBCs. A. Neutrophil containing bacterial bacilli, cat. B and C. Neutrophil (B) and monocyte/macrophage (C) containing nonstaining bacilli indicative of *Mycobacterium* sp., dog (from ASVCP slide contributed by Dr. H.W. Tvedten, 1988). D. Morula of *Ehrlichia ewingii* in a neutrophil, dog. E. Morula of *Anaplasma phagocytophilum* in a neutrophil; horse. F. Morula of *Ehrlichia canis* in a granular lymphocyte, Brazilian dog (blood film courtesy of Dr. Camilo Bulla, Michigan State University).

1. *Ehrlichia* spp. and the leukocytic *Anaplasma* spp. enter certain WBCs of many species and multiply within cytoplasmic vacuoles (Table 2.18). The resulting clusters of organisms (2 to > 20) are called *morulae* (singular morula, Latin for mulberry), which typically have diameters of 1.5–4.0 μm and stain pale to dark blue with a Romanowsky stain. In granulocytic forms, there may be more than one morula per WBC. Morulae should not be confused with Döhle bodies, platelets lying on WBCs, or blebs of nuclear membrane.
   a. *E. canis* morulae may be detected in monocytes or lymphocytes of blood from affected dogs or cats, particularly in endemic areas.[195–198] Rarely, morulae of *E. canis* have been reported within neutrophils and eosinophils.[199,200]
   b. In dogs infected with *E. ewingii*, morulae are present in 0.1–10 % of neutrophils in the first few days of illness but are rare thereafter.[201]
   c. Morulae are seen in many dogs, horses, ruminants, and cats infected with *A. phagocytophilum*, organisms commonly being present in 5–30 % of neutrophils within the first 10 d of infection.[202–205] Rarely, they have been reported in alpacas and llamas.[206]
   d. Morulae were not seen in a dog infected with Panola Mountain *Ehrlichia* sp.,[207] but rare morulae were seen in nongranulocytic cells of goats infected with this

Table 2.18. Major anaplasmal species that infect WBCs of domestic mammals[a]

Genus	Anaplasmal species	Hosts	Disease name	Cells infected
Ehrlichia	*E. canis*	Dogs	Canine ehrlichiosis (tropical pancytopenia)	Monocyte, lymphocyte (rarely granulocyte)
		Cats (rare)	Feline ehrlichiosis	
	E. ewingii[b]	Dogs	Canine granulocytic ehrlichiosis	Neutrophil, eosinophil, very rarely monocyte
Anaplasma	*A. phagocytophilum*[c]	Dogs	Canine granulocytic anaplasmosis	Neutrophil, eosinophil
		Cats	Feline granulocytic anaplasmosis	
		Horses	Equine granulocytic anaplasmosis	
		Cattle	Tick-borne fever or bovine, ovine, or caprine granulocytic anaplasmosis	
		Sheep		
		Goats		
		Llamas and alpacas	Granulocytic anaplasmosis	
Neorickettsia	*N. risticii*[d]	Horses Dogs (rare) Cats (rare)	Potomac horse fever	Monocyte
	N. helminthoeca[e]	Dogs	Salmon poisoning disease	Monocyte/macrophage

[a] This table includes major organisms in the family *Anaplasmataceae* (order *Rickettsiales*) that infect WBCs of dogs, cats, horses, cattle, llamas, or alpacas. Other cell types also may be infected. Canine infections with *E. chaffeensis*, *E. muris*, and Panola Mountain *Ehrlichia* sp. have been reported. Additionally: *Anaplasma bovis* infects monocytes in cattle (rare reports in horses and dogs), mostly in Africa, Asia, and South America; *Ehrlichia ruminantium* (basonym: *Cowdria ruminantium*) infects ruminant neutrophils and endothelial cells, primarily in Africa and parts of the Caribbean. Other anaplasmal agents infect other cells: *Anaplasma platys* (basonym: *Ehrlichia platys*) infects canine platelets, *Anaplasma* spp. infect ruminant RBCs, and *Neoehrlichia mikurensis* infects endothelial cells in dogs.

[b] *E. ewingii* and *A. phagocytophilum* infections in people have been reported.

[c] Basonyms: *Ehrlichia equi* and *Ehrlichia phagocytophila*

[d] Basonym: *Ehrlichia risticii*

[e] *Neorickettsia* sp. SF agent (so-named for its association with the fluke *Stellantchasmus falcatus*) has been associated with disease similar to salmon poisoning disease.

Note: Basonyms are the original official names prior to renaming. Recognizing basonyms helps understanding of older literature.

bacterium.[208] Canine infections with *E. muris* have been reported;[209] morulae would be expected in nongranulocytic WBCs.

2. For many years, anaplasmal agents that infected WBCs were classified within the genus of *Ehrlichia*. Molecular diagnostic methods including 16S rRNA analyses resulted in the reclassification of several organisms that previously were considered ehrlichial agents and that looked similar microscopically. Some were moved to the *Anaplasma* genus; others to *Neorickettsia*. The appropriateness of a nomenclature system purely dependent on such genetic sequences has been questioned.[210]
3. Anaplasmal species that infect domestic mammals are usually considered separate from those that infect people (*Ehrlichia chaffeensis* and *Neorickettsia sennetsu*), but human isolates have been found that could not be distinguished from *E. ewingii* by nucleic acid sequencing and PCR testing,[211,212] and *Ehrlichia chaffeensis* has been identified in dogs.[213] Also, agents previously thought to be separate species (*E. equi*, *E. phagocytophila*, and human granulocytic ehrlichia) are now considered to be one organism – called *Anaplasma phagocytophilum* – although there appear to be species variants based on the selective ability of various isolates to induce disease across species.[203,214,215] Genetic variants occur within single species and in the same geographic area.[204]

C. *Histoplasma capsulatum* (Figure 2.16A)

1. The yeast phase of *H. capsulatum* can be found singly or in multiples within the cytoplasm of monocytes (or macrophages), neutrophils, and occasionally eosinophils of blood. Cells containing *Histoplasma* may be more easily found at the sides or

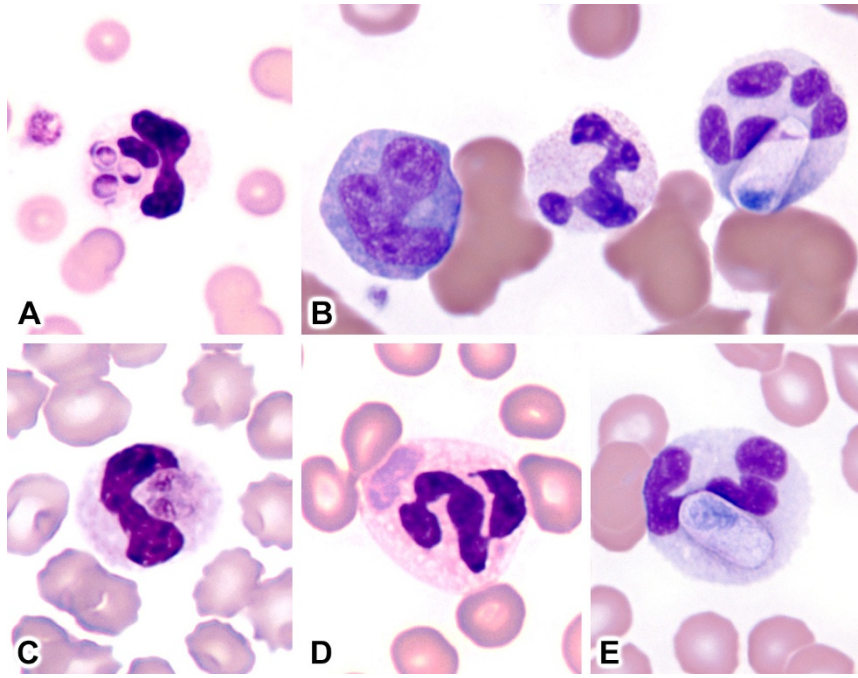


Figure 2.16. A. Yeast stages of *Histoplasma capsulatum* in a neutrophil, cat. B and E. Gametocytes of *Hepatozoon americanum* in monocytoid cells compared to uninfected monocyte and neutrophil (B), dog (from ASVCP slide contributed by Dr. C.J. LeBlanc et al., 2002). C. Tachyzoites of *Toxoplasma gondii* in a neutrophil, dog. Distemper inclusions in a neutrophil, dog (from ASVCP slide contributed by Dr. J.C. Tobey, 1993).

feathered end of a blood film (because larger cells are concentrated there) or in buffy coat preparations.

2. The organisms are round to oval, about 2–4 μm long, with a prominent cell wall. Their nuclei are commonly eccentric and stain pale blue and/or dark pink to purple with a Romanowsky stain. This appearance is not exclusive to *H. capsulatum*; *Histoplasma*-like yeast should also be considered.
3. When found in blood, the infection has advanced to disseminated histoplasmosis, and organisms can typically be found in macrophages of bone marrow, spleen, liver, lymph nodes, and other tissues.

D. *Hepatozoon americanum*[216–218] (Figure 2.16B and E)

1. This is a protozoon whose merozoites infect canine neutrophils or monocytes to form gamonts (also called gametocytes). Prior to 1997, the North American organism was classified as *H. canis*.[216] In North America, it is found with its tick hosts and vector mostly in the Southern or Gulf Coast states, but also in western, central, and northeastern states. It causes American canine hepatozoonosis, the clinical signs of which include fever, pain, lameness, and ocular discharge. Affected dogs often die within 1–2 yr if not treated, and although treatment is not curative, it can resolve clinical signs.
2. Finding gamonts of *H. americanum* in blood monocytes or neutrophils is very uncommon in clinical cases; when present, there is typically a very low percentage of infected WBCs. Gamonts are oval to elliptical, pale blue, and measure about 9 μm × 4 μm. They may fill a cell's cytoplasm and cause peripheral displacement of the cell's nucleus. The organism may escape from the cell and leave a nonstaining area surrounded by a capsule.
3. Encysted forms of the protozoa are common in skeletal muscle, and a skeletal muscle biopsy is one method of confirming a diagnosis.
4. Common laboratory data in clinical cases include neutrophilic leukocytosis (sometimes extreme and leukemoid), a normocytic normochromic nonregenerative anemia, thrombocytosis, increased serum alkaline phosphatase activity, hypoglycemia (which may be caused by glucose consumption by WBCs in vitro), hypoalbuminemia, and corresponding hypocalcemia. There is poor cross-reactivity of antibodies to *H. americanum* and antibodies to *H. canis*.

E. *Hepatozoon canis*[217,218]

1. This is a protozoon whose merozoites infect canine neutrophils or monocytes to produce gamonts (also called gametocytes). It can be difficult to determine if neutrophils or monocytes are infected, but findings suggest that only monocytes are infected in at least some cases.[219] It has a relatively worldwide distribution, including southeast and southcentral regions of the USA; several ticks are hosts and vectors of hepatozoonosis caused by this organism.
2. Infected dogs frequently lack obvious clinical signs but can be febrile, lethargic, anorexic, and very sick. In contrast to *H. americanum*, few to many gamonts may be found in blood WBCs, with sometimes a high percentage of the neutrophils or monocytes infected; this may vary by geographic region.[220–222] Via light microscopy, the gamonts are very similar to those of *H. americanum* except that *H. canis* structures are a little longer (measuring ~11 μm in length). Encysted forms of the protozoa are rare in skeletal muscle.

3. Microscopic examination of buffy coat smears has substantially greater diagnostic sensitivity for detecting gamonts than evaluation of blood smears, but PCR testing of blood and/or buffy coat samples is even more diagnostically sensitive.[158]

F. *Hepatozoon* spp. in cats:[223,224] Feline *Hepatozoon* infections have been reported in Asia, Africa, Europe, South America, and North America. They are typically subclinical with few gamonts in circulating neutrophils and monocytes. Three species have been reported to infect cats: *H. felis*, *H. canis*, and *H. silvestris*.

G. *Leishmania* spp.
   1. *Leishmania* is a kinetoplastid protozoon that is found primarily in Mediterranean, Central American, and South American countries. However, canine *L. infantum* infections have been found in many parts of North America, particularly in hunting dogs.[225–230] It can be transmitted vertically or via sandflies.
   2. Amastigotes, protozoal stages without flagellae, rarely are seen in circulating neutrophils, monocytes, or macrophages of cats or dogs with leishmaniasis.[231–233] They appear as 2–4 μm-long round or oval structures, each with a purplish round to oval nucleus and a prominent purplish cytoplasmic short bar called a *kinetoplast*, a specialized mitochondrial structure containing extranuclear DNA. *Leishmania* amastigotes must be differentiated from amastigotes of *Trypanosoma cruzi*, which also have a kinetoplast.
   3. Macrophages and neutrophils laden with amastigotes are usually numerous in bone marrow, lymph nodes, spleen, and liver. Amastigotes have been reported within bone marrow eosinophils and neutrophil precursors.[233]

H. *Toxoplasma gondii* (Figure 2.16C): Tachyzoites of *Toxoplasma gondii* are rarely found in blood neutrophils and monocytes of dogs and cats with acute toxoplasmosis. They are more common in macrophages of infected tissues (e.g, lung, intestine). They are crescent shaped, 5–8 μm long and 2–3 μm wide at their center with a relatively central purplish nucleus. However, they may be too packed together within cells to see their structure; organisms that are free or associated with lysed cells may be easiest to identify.

I. *Sarcocystis* spp.: Merozoites of a *Sarcocystis* protozoan were present extracellularly and in few lymphocytes, monocyte/macrophages, and neutrophils of a cat with a concurrent feline immunodeficiency virus infection.[234] The merozoites were pale blue elongate ovoid structures (5–7 μm × 1–2 μm), each with a central, purple nucleus. Merozoites of *Sarcocystis* were also found in bovine nongranulocytic WBCs during experimental infections.[235,236]

J. *Rangelia vitalii*: This tick-transmitted protozoon infects endothelial cells and can be seen in RBCs, neutrophils, and monocytes of dogs with acute rangeliosis in South America.[237]

K. Canine distemper inclusions (Figure 2.16D)
   1. Inclusions appear as red to purplish-red or pale blue cytoplasmic inclusions in neutrophils, monocytes, lymphocytes, and/or RBCs (see Chapter 3). Quick stains (e.g., Diff-Quik) stain the viral particles better (brighter) than Wright stains, which typically produce pale blue inclusions.
   2. Rather than viruses, they are aggregates of nucleocapsids that can develop into infectious virions in other cells. They typically occur in the early viremic stage and before clinical illness. They are rarely observed in regions with high rates of distemper vaccination (most of the United States) but are seen in regions where there are more unvaccinated dogs and more dog-to-dog contact.

III. Other acquired WBC inclusions

A. *Sideroleukocytes* are neutrophils or monocytes containing hemosiderin (Figure 2.14B). On Romanowsky-stained blood films, hemosiderin is a blue-green or yellow-brown to black pigment (golden brown unstained) that can be confirmed with an iron stain (Prussian blue). Sideroleukocytes occur with hemolytic anemias (e.g., equine infectious anemia or immune-mediated hemolytic anemia) and after transfusion, typically in low number. The hemosiderin may have been engulfed while the cells were in a spleen, liver, or other tissue.[238,239]
B. *Erythrophages* are monocytes or neutrophils that have engulfed one or more RBCs (Figure 2.14C). They are occasionally seen in immune hemolytic anemias such as idiopathic immune-mediated hemolytic anemia in dogs, equine infectious anemia, and neonatal isoerythrolysis.
C. *Lupus erythematosus (LE) cells* (very rare in blood) are neutrophils that contain an engulfed homogeneous mass of nuclear antigen-antibody complexes that typically compresses the nucleus peripherally. With a Romanowsky stain, this material usually appears paler and pinker than the adjacent nucleus but can rarely stain pale blue. LE cells are evidence of antinuclear antibodies and immune-mediated disease.
D. Neutrophils containing mast cell granules may be seen in blood smears of cats or dogs with systemic mast cell neoplasms, but rarely (Figure 2.17A and B). Few to many neutrophils may contain one to several purple granules of the same size and character as mast cell granules, but mast cells may or may not be present.[177,240,241]
E. Melanin granules have been reported in blood neutrophils of a horse with a disseminated malignant melanoma; pigmented cells suggestive of neoplastic melanoblasts were also present.[242]
F. Nuclear membrane blebs (vesicular appendages) are pale purple paranuclear veil-like bulges of the nuclear membrane that are not uncommonly present in neutrophils, particularly of healthy or sick cats. Although they have been associated with cobalamin deficiency, myeloid and lymphoid leukemias, chemotherapy, and radiation therapy in people, they commonly occur without these associations in veterinary medicine, where they have no known clinical significance.[243,244] Their location, shapes, and staining differentiate them from clinically significant inclusions, and they are typically not reported.
G. Nonstaining, distinct cytoplasmic vacuoles are common in monocytes and, to a lesser degree, neutrophils in blood smears made from EDTA-anticoagulated blood; these are routinely not reported. Monocytes typically lack vacuoles in smears made immediately

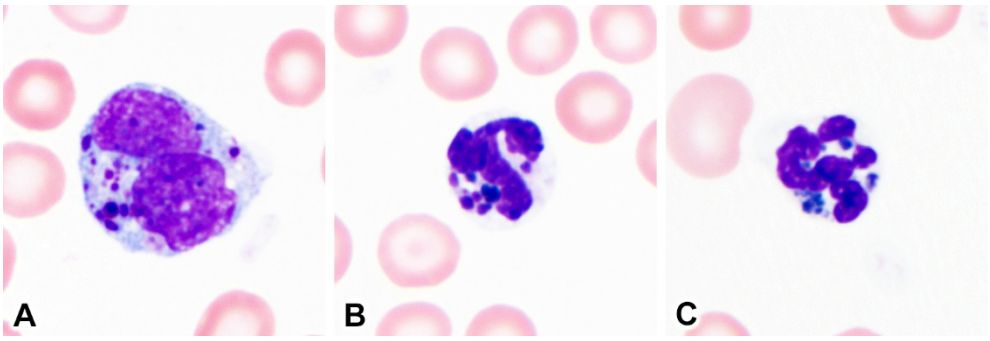


Figure 2.17. Purple mast cell granules in a monocyte (A) and neutrophil (B), dog with neoplastic mastocytemia. C. Greenish lipofuscin inclusions, neutrophil, dog with hepatic disease.

after blood collection, but vacuoles may develop in EDTA-anticoagulated blood by 30 min and become more numerous over time. Neutrophil vacuolation occurs more slowly and to a more limited degree; the distinct nature of these vacuoles distinguishes this in vitro change from the cytoplasmic clearing of toxic change, which is often called vacuolation and is typically associated with other toxic changes.

H. In Romanowsky-stained blood smears from occasional patients, clusters of greenish granular material typical of lipofuscin may be seen in a small percentage of neutrophils and monocytes, though it may not be reported (Figure 2.17C). In people, it has been associated with hepatic disease and a high mortality rate.[245] It also occurs in dogs with severe disease and hepatocellular damage.[246] Lipofuscin released from damaged hepatocytes may be engulfed by neutrophils and monocytes circulating through a diseased liver.

I. Other WBC inclusions: A variety of other cytoplasmic inclusions may be seen and reported, but they have no clear diagnostic value.
   1. Neutrophils may have prominent magenta granules in some foals and adult individuals of multiple species without an apparent clinical problem related to the neutrophils; this should be considered over toxic granulation when no other signs of toxic change are present.
   2. WBCs can contain detached Howell–Jolly-like nuclear bodies that in people have been associated with retroviral infections, primary or secondary myelodysplasia, and treatment with chemotherapeutic, immunosuppressive, and antiviral drugs.[247,248]
   3. Eosinophil granules are occasionally seen in equine neutrophils, apparently having been phagocytized.

IV. Hereditary disorders that have WBC inclusions (rare)

A. Lysosomal storage diseases: Lysosomes are membrane-bound, cytoplasmic, acidic organelles containing a variety of enzymes important in degradation of complex cellular or extracellular macromolecules. *Lysosomal storage diseases* are progressive inborn errors of metabolism that cause accumulation of incompletely degraded material within lysosomes of many cells, in some cases WBCs in blood and their precursors in bone marrow (Figure 2.18A–C).[249]
   1. The causative mutations occur in genes for lysosomal enzymes, lysosomal enzyme activator proteins, or proteins needed to transport material out of lysosomes.
   2. Several of the many known lysosomal storage diseases have been documented in domestic mammals and are typically diagnosed in young animals with developmental abnormalities or neurologic disease. Basic information about those which cause inclusions in blood WBCs are provided in Table 2.19.[249–257]
   3. Other storage diseases have been recognized in domestic mammals, but without WBC inclusions. These include MPS type IIIA in wirehaired dachshunds[258] and a New Zealand huntaway dog.[259]

B. Chédiak–Higashi syndrome[260,261] is a hereditary disorder that has been recognized in blue-smoke Persian cats, Hereford cattle, Brangus cattle, Japanese black cattle, and other nondomestic mammals. Diagnostic features include large purplish and eosinophilic (orangish) primary and secondary granules in the cytoplasm of neutrophils, eosinophils, and basophils. Abnormal granules reflect fusion of lysosomes caused by defects in the gene (*LYST*) for a lysosomal trafficking regulator protein involved in synthesis, transport, and fusion of cytoplasmic vesicles. Affected animals have hypopigmentation, impaired neutrophil function with lysosome content deficiencies (e.g., elastase), increased

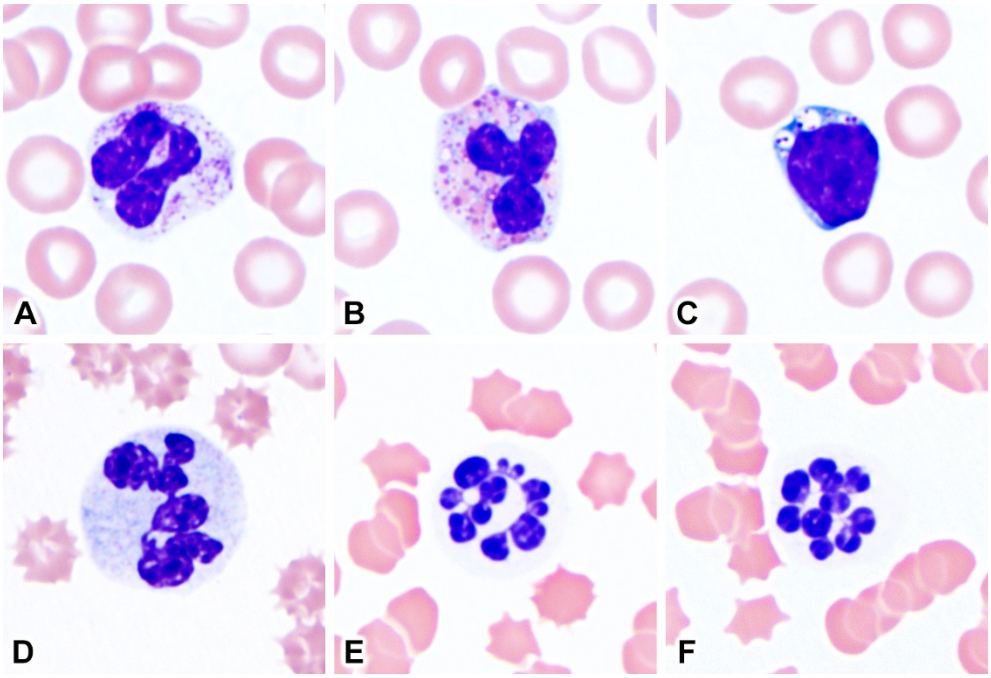


Figure 2.18. Abnormal inclusions in neutrophil (A), eosinophil (B), and lymphocyte (C) of a puppy with mucopolysaccharidosis VII. D. Giant hypersegmented neutrophil, Australian shepherd puppy with congenital cobalamin deficiency (previously published).[105] E and F. Idiopathic neutrophil hypersegmentation, quarter horse.

susceptibility to infection, and impaired platelet function causing a bleeding tendency. Chédiak–Higashi syndrome can be considered a lysosomal storage disease, but it is often discussed separately because it is a trafficking disorder, not a lysosomal enzyme defect.

C. May–Hegglin anomaly, caused by a mutation in the gene (*MYH9*) for the heavy chain of nonmuscle myosin heavy chain IIA, was diagnosed in an 8-yr-old pug.[262] As in humans affected by this heritable condition, the dog had macrothrombocytopenia and neutrophils containing one or more 2 × 4 μm fusiform to irregular grey-blue inclusions that were similar in color to Döhle bodies. The inclusions had positive immunolabeling with an antibody to nonmuscle myosin heavy chain IIA.[263] The dog was initially treated for a misdiagnosis of immune-mediated thrombocytopenia; it had no clinical signs attributable to its hematologic abnormalities.

D. Neutrophil granulation anomaly in Birman cats is a hereditary condition that is not associated with neutrophil dysfunction or clinical disease. Neutrophils have prominent, reddish, diffuse to somewhat patchy granulation caused by abnormal lysosome content that is not acid mucopolysaccharide. Similar red granules were described in basophils of one Birman cat, intermixed with abnormal pale to dark basophil granules.[264] Breed and clinical findings help exclude a similar-appearing mucopolysaccharidosis.

V. Pelger–Huët anomaly and inherited granulocyte hyposegmentation of Australian shepherds[265–271]

Table 2.19. Lysosomal storage diseases affecting circulating WBCs of domestic mammals

Disorder	Defective enzyme	Species	Blood cells	Expected cytoplasmic features[a]
GM1 gangliosidosis	β-Galactosidase	Cattle, cats, dogs	Lymphocyte, monocyte, neutrophil, eosinophil	Unstained vacuoles (if any inclusion)
GM2 gangliosidosis	β-N-Acetylhexosaminidase	Dogs, pigs, cats	Neutrophil, lymphocyte	Magenta to dark purple granules or vacuoles (some with stained core)
MPS type I	α-L-Iduronidase	Cats, dogs	Lymphocyte	Usually none seen
MPS type IIIB	α-N-Acetylglucosaminidase	Dogs	Lymphocyte	Magenta to purple granules
MPS type VI	Arylsulfatase B	Dogs, cats	Neutrophil, lymphocyte, monocyte, eosinophil, basophil	Coarse red to purple granules in granulocytes; similar granules or vacuoles in lymphocytes and monocytes
MPS type VII	β-Glucuronidase	Dogs, cats	Neutrophil, eosinophil, lymphocyte, monocyte	Magenta to purple granules; unstained halos around lymphocyte granules
Fucosidosis	α-L-Fucosidase	Dogs, a cat	Lymphocyte	Vacuoles
α-Mannosidosis[b]	α-Mannosidase	Cats, cattle	Lymphocyte, monocyte	Vacuoles

[a] Reports of light microscopy features are few, reported features have varied, and some features have been incompletely described or described with words that do not clearly reflect color, such as metachromatic, which means staining a color different from colors or color combinations of a stain's dyes, or azurophilic, which means staining with an azure dye but does not indicate what color combination of red and blue is present. Staining varies with stains and staining conditions, color varies with the density of inclusions, and similar inclusions can appear as vacuoles, vacuoles with stained cores, or granules depending on the cell or smear. Additionally, color perception varies among individuals.

[b] Findings are similar with β-mannosidosis in goats.

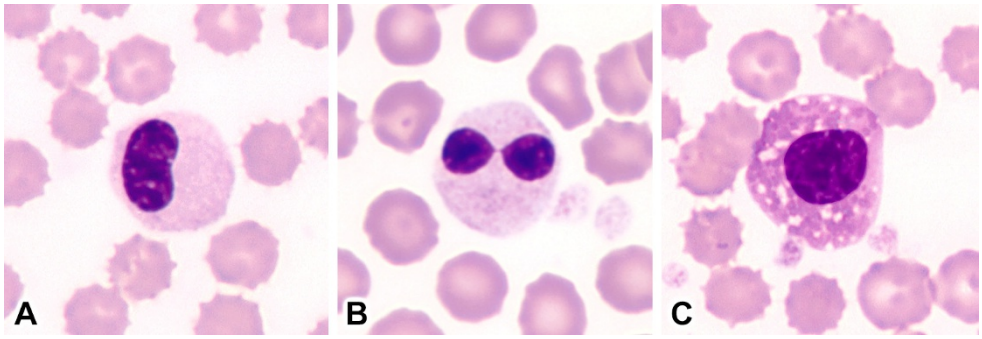


Figure 2.19. Congenital granulocyte hyposegmentation, dog. A. Short sausage-shaped nucleus with condensed chromatin, neutrophil. B. Dumbbell form of hyposegmentation, neutrophil. C. Round condensed nucleus, eosinophil.

A. In people, Pelger–Huët anomaly is a benign autosomal dominant disorder caused by mutations in the gene for the lamin B receptor, an important integral protein of the inner nuclear membrane. Heterozygotes have nuclear hyposegmentation, most prominent in neutrophils, but with little clinical consequence. Homozygotes develop skeletal disease and die in utero.
B. Similar hereditary disease with nuclear hyposegmentation has been reported in several breeds of dogs (including mixed breeds), domestic shorthair cats, and Arabian horses, but with little genetic investigation. Negative clinical outcomes have not been reported other than a stillborn homozygotic kitten.[266] The commonality is the characteristic hyposegmentation of neutrophil, eosinophil, and basophil nuclei. The nuclei are round, oval, spectacle shaped, or occasionally have partial segmentation (Figure 2.19). The nuclear chromatin may appear hyperchromatic or normochromatic but lacks the paler appearance of typical band neutrophils. The various nuclear shapes can be graded to characterize the degree of hyposegmentation.[269,272] In some animals, decreased lobulation of monocyte and megakaryocyte nuclei has been described.[272]
C. Inherited granulocyte hyposegmentation of Australian shepherds is relatively common and has long been referred to as Pelger–Huët anomaly, but it is not analogous to the human condition. Genetic testing does not support an autosomal dominant defect in the lamin B receptor gene, and instead supports an autosomal recessive disorder associated with a variant for *LMBR1L* (limb development membrane protein1 like), a gene encoding a protein without a known connection to nuclear hyposegmentation.[271]
D. Neutrophils of Pelger–Huët anomaly or other inherited granulocyte hyposegmentation disorders should not be classified as band neutrophils (or younger forms), because they appear to function like mature cells and do not indicate a left shift or inflammatory disease.

VI. Noninflammatory pseudo-Pelger–Huët anomaly: Like inflammatory pseudo-Pelger–Huët anomaly, noninflammatory pseudo-Pelger–Huët anomaly is acquired and characterized by WBC features mimicking Pelger–Huët anomaly (hyposegmented nuclei with clumped chromatin).
A. Certain drugs may cause transient pseudo-Pelger–Huët anomaly; in people, the drug-induced variant tends to yield mostly monolobate nuclei.[273]

B. Persistent pseudo-Pelger–Huët anomaly occurs with some myeloid leukemias, some myelodysplastic syndromes, and with ionizing radiation, each of which may cause mutations like those of Pelger–Huët anomaly.[274–276]

VII. Nuclear hypersegmentation unrelated to cell aging

A. Hyperthermia caused by heat stroke,[277] Adderall (a combination amphetamine) toxicity,[278] or other problems results in hypersegmentation of many WBC nuclei accompanied by inappropriate rubricytosis and other WBCs undergoing cell death (e.g., pyknotic nuclei) (Figure 3.9). Hypersegmentation is most apparent in neutrophils and eosinophils where it is characteristically botryoid, meaning that the nuclear lobes radiate out from center in thin stalks like a cluster of grapes.

B. Cobalamin (vitamin B_{12}) deficiency caused by a congenital cobalamin receptor defect leads to stunted growth and acquired myelodysplasia that can include hypersegmented neutrophil nuclei (Figure 2.18D).[105,279]

C. Idiopathic hypersegmented neutrophils occur in rare quarter horses.[56,280,281] In affected horses, most of the neutrophils are markedly hypersegmented with as many as 11 lobes separated by filaments (Figure 2.18E–F). The condition is not associated with clinical disease. In the authors' experience, it can be challenging to recognize a left shift in affected horses, but the presence of toxic change is helpful to indicate the presence of inflammation.

D. Bone marrow dyscrasia of poodles is a hereditary disorder seen in miniature and toy poodles.[282,283] The developmental defect in cell production is not known, and there is not a clinical problem associated with the syndrome. Diagnostic features are hypersegmented and/or giant neutrophils and macrocytic normochromic RBCs without anemia and typically without reticulocytosis. RBC mean cell volumes usually are 80–110 fL compared to 60–77 fL in most unaffected dogs. A potential variant with reticulocytosis and otherwise similar features has been reported.[284]

E. Other: Myelodysplastic syndromes and myeloid leukemias involving the neutrophil series may yield atypical cells including normocytic or giant hypersegmented or normosegmented neutrophils.[285] Giant hypersegmented neutrophils have been observed in dogs after chemotherapy including cyclophosphamide treatments (authors' observation) and experimentally in rats.[286]

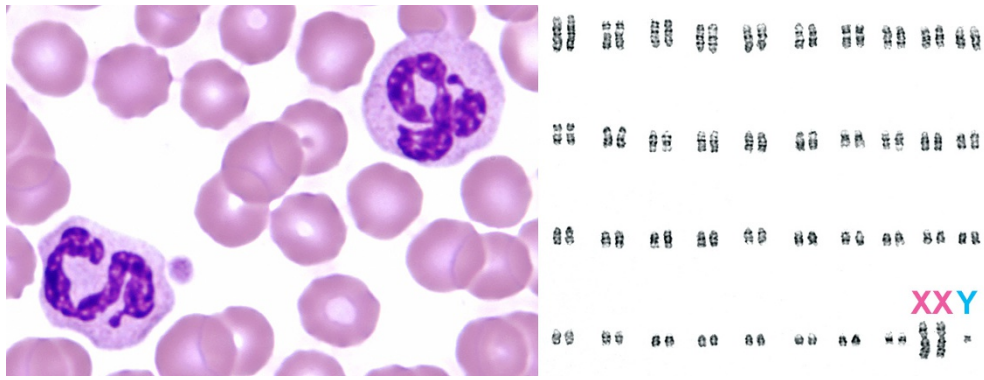


Figure 2.20. Neutrophils with Barr bodies (*left*) in a 3-mo-old phenotypically male greyhound-English pointer with a 79XXY karyotype (*right*) indicative of Klinefelter's syndrome (note the X, X, and Y chromosomes *in the lower right corner*); 16 % of the neutrophils contained a Barr body. Clinical information and karyotyping courtesy of Drs. Dennis O'Brien and Gary Johnson, University of Missouri–Columbia.

VIII. Barr bodies associated with chromosomal abnormalities (Figure 2.20)
- A. A Barr body is a mass of condensed chromatin containing the inactivated X chromosome of somatic cells in female mammals. In health, a Barr body is apparent in a minority of neutrophils, appearing as a drumstick (club) attached to a terminal nuclear lobe by a thin nuclear filament. Similar morphologic structures, not necessarily on terminal lobes, mimic Barr bodies in females and males.
- B. Prominence of Barr bodies in an animal thought to be male suggests Klinefelter's syndrome (XXY) or a mosaic of XXY and XY karyotypes (Figure 2.20).[287]
- C. The presence of two Barr bodies in multiple neutrophils suggests trisomy X or a mosaic disorder including trisomy X, with inactivation of two X chromosomes per cell.[288]

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 3

ERYTHROCYTES

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Table 3.1. Abbreviations and symbols in this chapter

[x]	x concentration (x = analyte)
2,3-DPG	2, 3-Diphosphoglycerate
AID	Anemia of inflammatory disease
ATP	Adenosine triphosphate
Bc	Conjugated bilirubin
Bu	Unconjugated bilirubin
C3	Complement protein 3
Cb_5R	Cytochrome-b_5 reductase (EC 1.6.2.2)
CBC	Complete blood count
CH	Cell (corpuscular) hemoglobin content mean
CHCM	Cell (corpuscular) hemoglobin concentration mean
CHCMr	Cell (corpuscular) hemoglobin concentration mean of reticulocytes
CHDWr	Cell (corpuscular) hemoglobin distribution width of reticulocytes
CHr	Cell (corpuscular) hemoglobin content mean of reticulocytes
CRP	Corrected reticulocyte percentage
DEA	Dog erythrocyte antigen
DNA	Deoxyribonucleic acid
EC	Enzyme Commission (followed by unique identifier numbers)
ECF	Extracellular fluid
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine infectious anemia virus
Epo	Erythropoietin
ESAIg	Erythrocyte surface-associated immunoglobulin (any type)
FAD	Flavin adenine dinucleotide
Fe	Iron, either ferrous (Fe^{2+}) or ferric (Fe^{3+})
FeLV	Feline leukemia virus
G6PD	Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
GLUT	Glucose transporter
GR	Glutathione reductase, glutathione-disulfide reductase (EC 1.8.1.7)

Table 3.1. Abbreviations and symbols in this chapter (Continued)	
GSH	Glutathione, reduced
HC	Hemoglobin concentration (in individual RBCs)
HCO_3^-	Bicarbonate
Hct	Hematocrit
HDW	Hemoglobin concentration distribution width
HDWr	Hemoglobin concentration distribution width of reticulocytes
Hgb	Hemoglobin (iron in Fe^{2+} state)
Hgb_{delta}	Hemoglobin delta (measure of free plasma [Hgb] by the ADVIA)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-x	Interleukin 1, 6, 8
IMHA	Immune-mediated hemolytic anemia
MCH	Mean cell (corpuscular) hemoglobin
MCHC	Mean cell (corpuscular) hemoglobin concentration
MCV	Mean cell (corpuscular) volume
MCVr	Mean cell (corpuscular) volume of reticulocytes
MetHgb	Methemoglobin
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NI	Neonatal isoerythrolysis
NMB	New methylene blue
nRBC	Nucleated red blood cell (nucleated erythrocyte)
PCR	Polymerase chain reaction
PCV	Packed cell volume
PFK	6-Phosphofructokinase (EC 2.7.1.11)
pH	–log $[H^+]$
PIMA	Precursor-targeted immune-mediated anemia
PK	Pyruvate kinase (EC 2.7.1.40)
RBC	Red blood cell (erythrocyte)
RC	Reticulocyte concentration
RDW	RBC distribution width
RDWr	RBC distribution width of reticulocytes
RI	Reference interval
RMT	Reticulocyte maturation time
RNA	Ribonucleic acid
RP	Reticulocyte percentage
SD	Standard deviation
SI	Système international d'unités (International System of Units)
TIBC	Total iron-binding capacity
TNF	Tumor necrosis factor
UIBC	Unbound (unsaturated) iron-binding capacity
WBC	White blood cell (leukocyte)
WRI	Within reference interval

Note: See the figure legends for abbreviations that are unique to the figures.

PHYSIOLOGIC PROCESSES

I. *Erythron*: all erythroid cells in an animal, including precursors and RBCs in blood vessels and sinuses of spleen, liver, and marrow (Figure 3.1)
   A. RBC precursors
      1. Erythropoiesis, the production of RBCs, is part of hematopoiesis, a complex system involving stem cells and cytokines. Several cytokines work together with Epo to stimulate the replication and differentiation of progenitor cells to committed stem cells that respond to Epo by either dividing or differentiating toward rubriblasts. Epo also promotes erythropoiesis by inhibiting apoptosis.
      2. Epo is produced mostly by the fetal liver and the adult kidney. Renal interstitial cells produce Epo in response to renal hypoxia, which may be caused by anemia, poor oxygenation of blood (e.g., high altitude or pulmonary disease), or poor renal perfusion.
         a. Hypoxia allows hypoxia-inducible factor 2α (HIF-2α) to translocate to the nucleus, bind to HIF-1β, and thus activate Epo gene transcription.

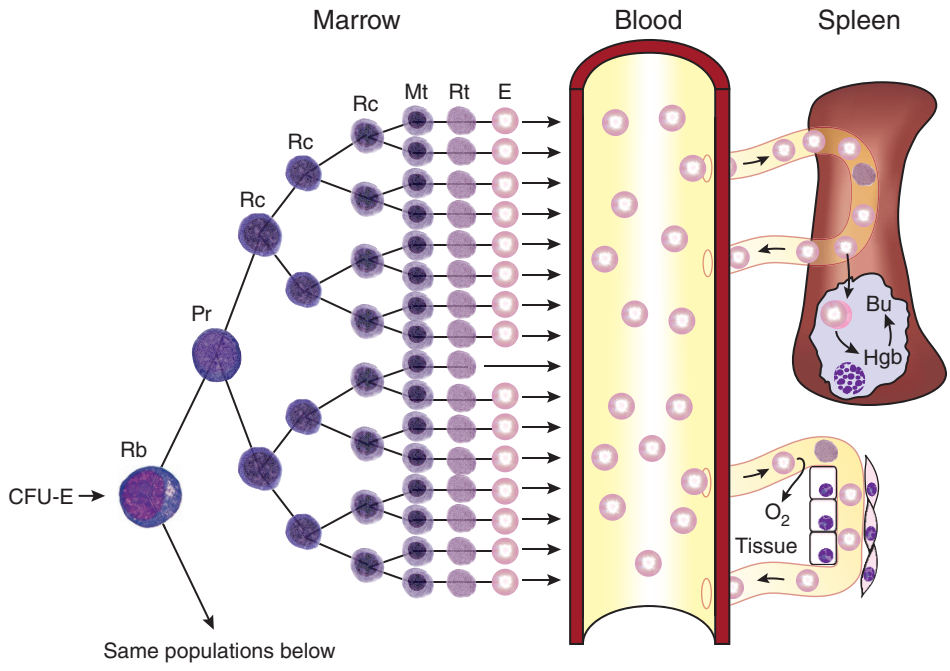


Figure 3.1. RBC kinetics in health. The erythron contains three major pools: (i) RBC progenitors and precursors (mostly in marrow), (ii) blood RBCs, and (iii) splenic RBCs. After stimulation by Epo, colony-forming unit–erythroid cells (CFU-E) differentiate into rubriblasts (Rb), and the precursors proliferate (via mitosis) and mature until erythrocytes (Es) are formed. An orderly maturation process produces a somewhat pyramidal distribution of erythroid cell populations (only the top half is shown). After release to the blood, RBCs circulate in the vascular system to transport O_2 to tissues and CO_2 to lungs. A pool of RBCs is in the spleen of most mammals. Senescent RBCs are destroyed by splenic macrophages, yielding Bu from Hgb.
Mt, metarubricyte; Pr, prorubricyte; Rc, rubricyte; and Rt, reticulocyte

b. In health, less HIF-2α reaches the nucleus to promote Epo production because, with greater O_2 levels, HIF-2α is kept hydroxylated by prolyl hydroxylase domain (PDH) enzymes, allowing it to associate with von Hippel Lindau (VHL) tumor suppressor protein and undergo enzymatic degradation. Mutations in genes for PDH, HIF, or VHL proteins can dysregulate erythropoiesis and cause erythrocytosis.

3. After a progenitor cell (CFU-E) differentiates into a rubriblast, two major cell processes occur:
   a. Cells undergo mitoses to produce more and smaller cells. Evidence suggests the increasing [Hgb] in developing cells decreases DNA synthesis and eventually limits mitosis.[1]
   b. Cells produce messenger RNA for synthesis of Hgb and cytoskeletal proteins.
4. RBCs develop in association with a macrophage (nurse cell), which is thought to provide signals, growth factors, and likely Fe to the erythroid precursor for Hgb synthesis. In the later stages of mammalian erythropoiesis, nRBC nuclei are extruded and engulfed by macrophages. The resulting anucleate cell is a *reticulocyte* if it contains stainable RNA.
5. When a nRBC loses its nucleus and ability to produce mRNA for protein synthesis, it soon loses the ability to produce Hgb and enzymes. Activities of RBC enzymes are greatest in reticulocytes and decrease slowly with age. Mitochondria deteriorate so that glycolysis becomes the ATP-producing pathway (except in pigs, which use an alternate energy pathway involving inosine).

B. Blood RBCs

1. Major features of RBCs in different species are compared in Table 3.2. In contrast to other domestic mammals, camelid RBCs are thin ellipsoid cells that contain greater concentrations of Hgb. Their thinness promotes gas exchange, which, in other species, is promoted by RBC biconcavity and a related high surface area to volume ratio. They have decreased deformability which may relate to different membrane composition and

Table 3.2. Comparison of typical blood RBCs in mature healthy animals

	Dogs	Cats	Horses	Cattle	Alpacas/ Llamas
RC ($\times 10^3/\mu L$)	50	40[a]	0	0	50 or 140[b]
[RBC] ($\times 10^6/\mu L$)	7	7–8	9	7	14
RBC life span (d)	100	70	150	150	60[b]
RBCs in blood[c] ($\times 10^{12}$)	5.6	2.2	450	280	82/200[d]
RBC diameter[e] (μm)	7	6	5–6	5–6	3.5 × 7
RBC volume (fL)	70	45	45	50	25
RBC central pallor	Prominent	Mild	None to mild	Mild to mod.	None

[a] Counting only aggregate reticulocytes
[b] Assessment of high-altitude animals yielded the higher RC and the RBC lifespan.
[c] Estimates based on 40 mL blood/lb body weight and average-sized animals
[d] Llamas have more circulating RBCs because they are larger than alpacas.
[e] Diameter (width × length for camelid elliptocytes) of spread RBCs in optimal regions of a blood smear; dimensions are lower for RBCs in suspension and substantially lower in formalin-fixed tissue sections (cell shrinkage)
Note: Individuals vary. All numbers represent approximate averages to illustrate similarities and differences.

cytoskeletal interactions, but they can swell substantially and are therefore resistant to hypotonic lysis as might otherwise occur with rehydration after periods of dehydration.

2. RBC destruction in health (senescence)
   a. About 200–250 million RBCs die per hour per kg body weight!
   b. As RBCs age, their enzyme activities decrease, they accumulate oxidative damage, lose membrane via vesiculation, and become more rigid, spheroid, and less capable of passing through splenic slit pores. Membrane antigens appear (e.g., on clusters of band 3 protein), and they are bound by naturally occurring antibodies that activate complement. This opsonization and other membrane changes help mediate phagocytosis of senescent RBCs by macrophages.[2,3]

C. Splenic RBCs
   1. Spleens of dogs, horses, and cattle have sinusoids and red pulp that are full of RBCs; 50–60 % of a horse's RBCs can be within its spleen. Damaged or less deformable RBCs are removed by macrophages that are adjacent to sinusoids or present in the red pulp. When splenic contraction occurs, RBCs are forced into the systemic blood.
   2. Cat spleens lack sinuses and have open blood circulation without the arteriovenous connections seen in dogs.[4,5] RBCs appear to pass from red pulp into the venous circulation through open-ended pulp venules rather than through narrow interendothelial sinus slits that, in other species, are important for removal ("pitting") of RBC inclusions such as Howell–Jolly bodies, Heinz bodies, siderotic granules, and intracellular organisms.[6,7] These slits also promote removal of poorly deformable RBCs. Less-restricted RBC passage through the feline spleen may also reduce remodeling of reticulocytes and explain the prolonged nonreticulocyte macrocytosis described in cats after regenerative anemia.[6,8,9]
   3. Most metarubricytes and reticulocytes that are released from marrow each day are temporarily trapped in mammalian spleens.

II. RBC kinetics
   A. A blood [RBC] is established by the relative rates of RBC production, shifting of RBCs to and from splenic sinuses, and RBC destruction.
   B. RBC production depends on the degree and duration of Epo stimulus and the capability of precursor cells to respond to Epo.

III. Hgb structure, function, synthesis, and degradation
   A. Hgb structure
      1. *Hgb* is a tetramer with each globin (polypeptide chain of amino acids) linked to a separate heme that binds O_2; its relative molecular mass is near 64,000. In mature healthy mammals, each Hgb molecule contains two α-chain and two β-chain globins. Approximately 95 % of a RBC is Hgb on a dry-weight basis, there being tens to hundreds of millions of Hgb molecules per RBC.
         a. Rates of synthesis of heme and globin are balanced and regulated by each other. If a precursor has very little heme and Fe^{2+} is available, heme and globin synthesis should increase.
         b. If synthesis of a globin chain is decreased, an animal has *thalassemia*.
         c. If gene mutations produce abnormal amino acid sequences in the globins, an animal has a *hemoglobinopathy*.
      2. Fe in heme is in the ferrous state (Fe^{2+}) and is kept in this reduced state by enzymatic reactions catalyzed by Cb_5R (NADH-methemoglobin reductase, commonly called

methemoglobin reductase) and NADPH diaphorase (NADPH-methemoglobin reductase).

3. Amino acids in the globin chains are maintained in a reduced state by reductive reactions involving GR and catalase.

B. Hgb function (Figure 10.3)
   1. Hgb transports O_2 from lungs to tissues. In health, Hgb is nearly 100 % saturated with O_2 in arterial blood. MetHgb (with Fe^{3+}) does not transport O_2.
   2. Hgb plays two major roles in the transport of CO_2 from tissues to lungs:
      a. When CO_2 diffuses into RBCs, carbonic anhydrase catalyzes its reaction with H_2O to form H^+ and HCO_3^-. Hgb acts as a buffer ($H^+ + Hgb^- \rightarrow HHgb$) to remove the H^+, and HCO_3^- diffuses from the cell to the plasma. The buffering of H^+ by Hgb facilitates the additional conversion of CO_2 to HCO_3^-. When RBCs return to lungs, reactions are reversed, and CO_2 is released for expiration. About 70 % of the CO_2 formed in tissues is transported to lungs via this system.
      b. When CO_2 diffuses into RBCs, some binds with Hgb to form carbaminohemoglobin. About 20 % of the CO_2 formed in tissues is transported to lungs via this system.

C. Hgb synthesis
   1. This occurs in RBC precursors (rubriblasts through reticulocytes) in a series of reactions (Figure 3.2).
   2. 5-Aminolevulinate synthase, porphobilinogen synthase, ferrochelatase, and coproporphyrinogen oxidase are inhibited by lead.
      a. In lead toxicity, inhibition of these enzymes leads to greater concentrations of heme precursors in RBCs. Collectively, porphobilinogen through protoporphyrin IX are called *porphyrins*.
      b. *Porphyria* is a condition in which concentrations of porphyrins in RBCs, plasma, or urine are increased. It can be acquired, as in lead toxicity, or congenital. Animals with porphyria are prone to photosensitivity.
   3. The maturation rate of RBC precursors is affected by cytoplasmic [Hgb]. If Hgb synthesis is incomplete, additional mitoses during a cell's development produce smaller RBCs.[1]

D. The type of Hgb synthesized in an individual can vary with the animal's age and health. In people, there are three major types: (i) embryonal Hgb with mostly two α globins and two ε globins, (ii) fetal Hgb with two α globins and two γ globins, and (iii) adult Hgb with mostly two α globins and two β globins. Embryonal Hgb is replaced by adult Hgb during fetal development in dogs, cats, and horses; these animals do not have fetal Hgb. Neonatal ruminants are thought to have a mixture of fetal Hgb (with two α globins and two βF globins) and adult Hgb, but only adult Hgb with two α globins and two βA globins after a few postnatal months.[10]

E. Hgb degradation and bilirubin metabolism: After RBC death in macrophages of spleen, liver, or marrow, the heme portion of Hgb is degraded in macrophages to biliverdin and then Bu, with heme Fe^{2+} being recycled and one carbon monoxide molecule being generated from each heme group. The globin portion of Hgb is degraded to amino acids, which also can be recycled. Bu is conjugated to Bc, which is excreted (Figure 3.3). The carbon monoxide gas diffuses to plasma and then into RBCs where it forms carboxyhemoglobin; it is later released in the lungs and exhaled. Because of this metabolism, carboxyhemoglobinemia commonly occurs with hemolytic anemia and may occur with internal hemorrhage.

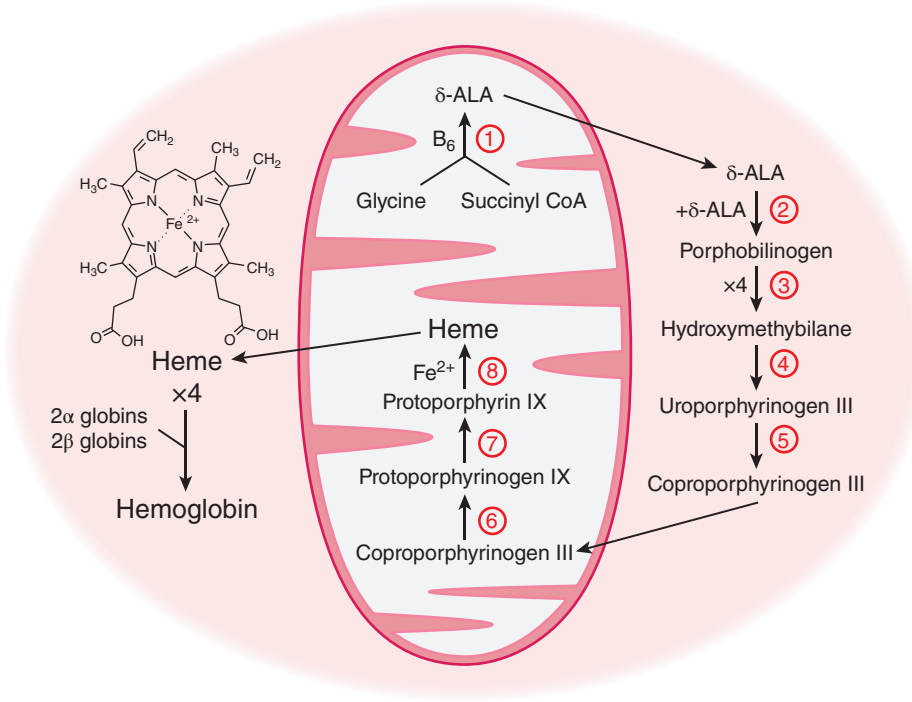


Figure 3.2. Hemoglobin synthesis in adult domestic mammals.

- Hgb synthesis in RBC precursors requires mitochondria and involves eight enzymatic reactions in which individual pyrrole rings are formed (steps ① and ②), polymerized into tetrapyrrole rings (steps ③ and ④), and modified (steps ⑤–⑦) prior to incorporation of Fe^{2+} into protoporphyrin IX to form heme which exits the mitochondrion. Each of four heme molecules then binds to one of two α or two β protein subunits to form tetrameric Hgb with four O_2 binding sites; this might involve the oxidized (Fe^{3+}) form of heme (ferriheme, hemin) forming MetHgb (not shown), which is reduced to Hgb. Reaction 1 is the rate-limiting step in heme synthesis; it requires vitamin B_6 (pyridoxine) and is inhibited by decreased Fe availability and greater cellular heme concentrations.
- Hereditary or acquired deficiencies in one or more of the eight enzymes in the Hgb synthesis pathway cause porphyria. The enzymes for each reaction are as follows: ① 5-aminolevulinate synthase, ② porphobilinogen synthase, ③ hydroxymethylbilane synthase, ④ uroporphyrinogen III synthase, ⑤ uroporphyrinogen decarboxylase, ⑥ coproporphyrinogen oxidase, ⑦ protoporphyrinogen oxidase, and ⑧ ferrochelatase.

δ-ALA, delta aminolevulinic acid; succinyl CoA, succinyl coenzyme A

IV. Fe

A. Total body Fe is distributed in three major sites in health: (i) about 50–70 % in RBC Hgb, (ii) about 25–40 % in storage (young animals have little stored Fe), and (iii) the remainder in other molecules (e.g., myoglobin, cytochromes, and enzymes). The very small fraction that circulates in plasma as transferrin does not reflect total body stores. Fe-binding proteins are important in limiting free Fe and associated oxidative injury while allowing for biological availability of Fe where it is needed.

B. Physiologic processes or concepts are provided in Figure 3.4.

C. In most mammals, the rate of intestinal absorption is influenced by the need for Fe by the body, i.e., if Fe is needed, more Fe is absorbed. In healthy adults, the amount of Fe absorbed per day is a very small percentage of total body Fe stores. A much greater amount of

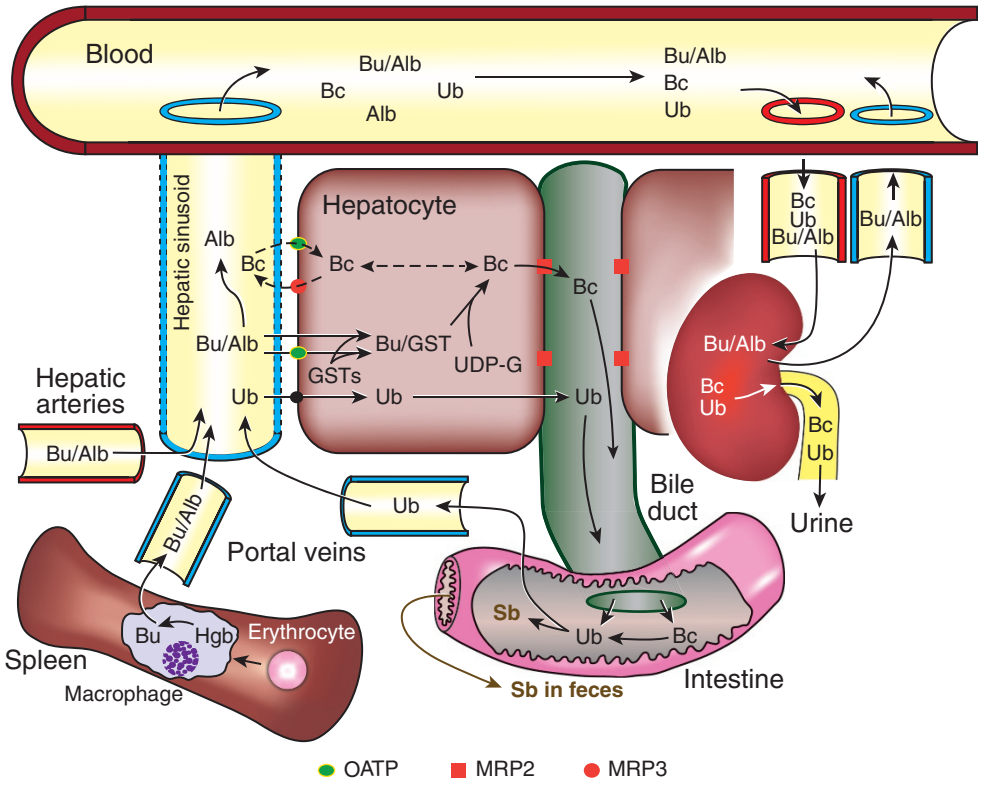


Figure 3.3. Physiologic processes involving bilirubin.

- RBC destruction within macrophages of the spleen (shown here), liver, or bone marrow is followed by the degradation of heme and its conversion to Bu. Clinically insignificant amounts of Bu are formed from heme degradation associated with ineffective erythropoiesis and degradation of other heme-containing molecules (catalase, peroxidase, and cytochromes). As Bu leaves a macrophage, it forms a noncovalent association with albumin (Alb) and remains associated with Alb until uptake by hepatocytes. Bu is relatively water insoluble prior to binding to Alb.
- When Bu enters the liver and its protein-permeable sinusoids, it probably binds to hepatocyte membrane receptors of the organic anion transporting polypeptide (OATP) family, enters hepatocytes without Alb, and binds to Y-protein (ligandin), a glutathione transferase (GST for glutathione S-transferase) or to Z-protein (a fatty acid–binding protein). This entry of Bu into hepatocytes is a passive but facilitated process; binding proteins enhance the process by reducing the efflux of Bu back to sinusoidal plasma.
- Within hepatocytes, Bu diffuses to the endoplasmic reticulum, where it is conjugated with glucuronide (or > 60 % with glucose in horses) to form bilirubin monoglucoronide or bilirubin diglucuronide, collectively called Bc. Conjugation makes the molecule more water soluble, and it diffuses to the canalicular membrane.
- Bc is transported from hepatocytes into canaliculi (the rate-limiting step in bilirubin excretion) by an energy-dependent transport system for organic anions other than BAs. The primary transporter is probably the multidrug resistance-associated protein 2 (MRP2), also called the canalicular multispecific organic anion transporter (cMOAT). There is evidence that some Bc moves from hepatocytes to sinusoidal blood (via MRP3) and back to hepatocytes via OATPs before secretion.[11]
- Bc in bile enters the intestine and is degraded to urobilinogen Ub, which is colorless. Urobilinogen can be passively absorbed in the intestine and then enter hepatocytes for excretion in bile or bypass the liver and be excreted in urine. Urobilinogen can also be degraded to stercobilinogen (Sb), which is dark brown and excreted in feces.
- Bc that passes from hepatocytes to blood without reuptake can pass through the glomerular filtration barrier and be excreted in urine. Because Alb does not pass through the glomerular filtration barrier of most mammals, Bu/Alb does not enter the urine in those animals.

Bu/Alb, Bu associated with albumin; UDP-G, uridine diphosphoglucuronide

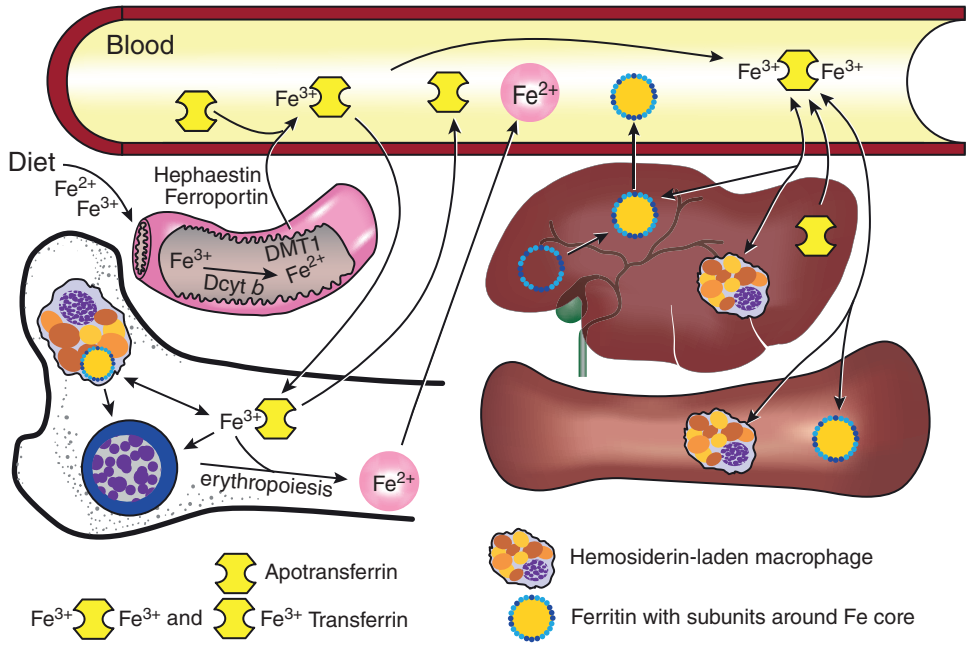


Figure 3.4. Fe kinetics in healthy animals.

- Absorption: Diets of domestic mammals may contain Fe^{2+} or Fe^{3+}. Ingested Fe^{3+} is converted to Fe^{2+} by ferric reductase (duodenal cytochrome *b* or Dcyt *b*), a surface enzyme, prior to entering enterocytes via divalent metal transporter 1 (DMT1). The Fe^{2+} is transported to plasma transferrin in the form of Fe^{3+} by ferroportin and hephaestin.
- Transport: Nearly all Fe in plasma is bound to apotransferrin, a transport protein (β globulin) produced by hepatocytes. When Fe is bound to apotransferrin, the complex is called transferrin. Transferrin carries Fe^{3+} to and from tissues, for use by cells or for storage. In health, about one-third of transferrin's Fe-binding sites are occupied by Fe. Many cells have transferrin receptors; importantly, these include erythroid precursors.
- Use in erythroid precursors: After transferrin binds to and enters erythroid precursors, Fe^{3+} dissociates from apotransferrin to ferritin or is incorporated into heme (Fe^{2+}) and then hemoglobin. Most apotransferrin escapes degradation and returns to plasma. Erythroid precursors may also acquire Fe from nurse macrophages, which have several mechanisms of obtaining Fe, including via transferrin.
- Storage: Fe^{3+} is stored in two protein-Fe complexes: ferritin (plasma and tissue) and hemosiderin (tissue macrophages).
  - Ferritin consists of apoferritin complexed with Fe^{3+} and is a relatively soluble, mobile source of Fe^{3+}. There are several forms of apoferritin because of various combinations of H or L subunits. Plasma ferritin is a glycosylated polymer that is relatively Fe poor. Tissue ferritin, which is nonglycosylated and relatively Fe rich, is produced by many cells, primarily macrophages, hepatocytes, intestinal mucosal epithelial cells, and erythroid precursors. Synthesis of apoferritin by hepatocytes and macrophages is increased by inflammation and when Fe storage is increased. Apoferritin is a positive acute-phase protein, measured and reported as [ferritin].
  - Hemosiderin is a relatively insoluble, poorly mobile source of Fe^{3+} and represents a major storage form of Fe that increases with excess iron. Hemosiderin is a complex of protein and Fe oxides that is found in lysosomes of macrophages. Ferritin is a source of Fe in hemosiderin, and Fe stored in hemosiderin can be transferred to ferritin for use. Hemosiderin-containing macrophages are found in the spleen, liver, and marrow of most mammals, but a healthy cat's marrow does not contain hemosiderin, at least not enough to be detected by routine stains for Fe.
- Tissue forms: A relatively small quantity of Fe is present in myoglobin, catalase, peroxidases, and cytochromes in addition to ferritin and hemosiderin.

available Fe comes from the ongoing degradation of senescent RBCs, each containing many millions of Fe atoms; this provides a continuous supply of recycled Fe, most of it being used for Hgb synthesis in erythroid precursors.

D. The intestinal absorption and cellular release of Fe are regulated by *hepcidin*, a small peptide hormone that is synthesized primarily by hepatocytes. Canine hepcidin has a molecular structure similar to that of human hepcidin.[12]
   1. If there are low concentrations of hepcidin within enterocytes, there is increased synthesis of the membrane Fe transporter ferroportin, which, in conjunction with hephaestin, a copper-containing Fe oxidase related to ceruloplasmin, transports the Fe^{2+} to plasma transferrin in the form of Fe^{3+}.
   2. In contrast, increased intracellular [hepcidin] decreases functional membrane ferroportin and also causes degradation of the apical Fe transporter. Therefore, less Fe is absorbed by intestinal epithelial cells and less can move from enterocytes to plasma; unabsorbed Fe within enterocytes is lost when mucosal cells are sloughed and replaced.
   3. The effect of hepcidin on macrophages is similarly to block and internalize ferroportin such that Fe export is reduced, e.g., to nRBCs for Hgb synthesis.

E. The synthesis of hepcidin is increased with increased Fe availability or increased IL-6 production during inflammation, and it is decreased indirectly by increased [Epo].[13]
   1. Increased tissue Fe stores and increased plasma [Fe] (transferrin-bound Fe) are sensed in the liver and lead to upregulation of hepcidin production by hepatocytes, thus decreasing Fe absorption when it is not needed. Fe deficiency decreases hepcidin synthesis, thus increasing Fe absorption when needed.
   2. Anemia or other causes of renal hypoxia cause increased plasma [Epo]. Epo stimulates erythroid precursors to produce erythroferrone, a hormone that downregulates hepatocyte production of hepcidin and thus makes Fe more available for erythropoiesis. With increased erythropoiesis, circulating [Fe] decreases, which also reduces signaling for hepcidin production. The result is increased movement of Fe from enterocytes to plasma and increased release of Fe from macrophages to plasma and RBC precursors.
   3. During inflammation, cytokines are released, including IL-6. IL-6 promotes the synthesis of hepcidin, which reduces ferroportin-mediated export of Fe from cells, including macrophages. This tends to sequester Fe in macrophages, and thus it is less available for erythropoiesis and for infectious agents.

V. Mature RBC metabolism (Figure 3.5)

A. Glucose is the major energy source for mature RBCs in most species. It enters RBCs by facilitated diffusion via GLUT1 or GLUT4. GLUT1 functions independently of insulin and also transports oxidized ascorbic acid into RBCs, where it is reduced to the antioxidant ascorbic acid (vitamin C). GLUT1 is expressed by fetal and neonatal RBCs, by RBCs in mammals that cannot synthesize ascorbic acid (guinea pigs, many primates, bats), and in adults of some breeds of dogs (Akitas and many shiba inus).[15,16] GLUT4 is expressed on RBCs of Akitas and at least some western breeds (e.g., beagles),[17] but little information on RBC GLUT expression in dogs is available. Whether insulin affects RBC GLUT4 activity, as it does for adipocytes and myocytes, is unknown.

B. Porcine RBCs lack a functional glucose transporter and use inosine instead of glucose.

VI. Reticulocytes

A. *Reticulocytes* are nonnucleated, immature RBCs with stainable cytoplasmic RNA. In camelids, they are commonly less elliptical than mature RBCs, or even round.

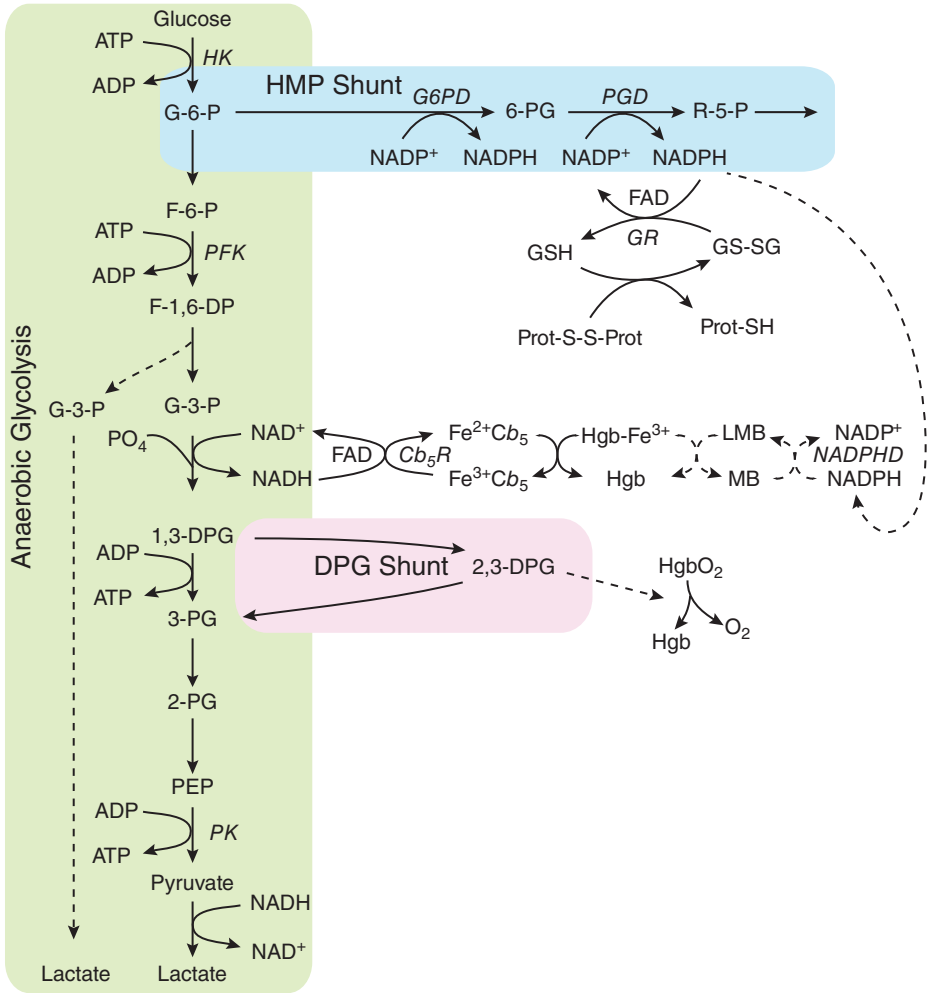


Figure 3.5. Major biochemical reactions in RBCs.

- Mature RBCs lack mitochondria and therefore glycolysis (*green background*; also known as the Embden–Meyerhof pathway) provides the biochemical skeleton for RBC metabolism and generates ATP and NADH. Because of the split of F-1,6-DP into two molecules of G-3-P for two parallel pathways, glycolysis consumes two molecules of ATP and produces four molecules of ATP. PFK is the rate-limiting enzyme, and its activity is enhanced by alkalemia and reduced by acidemia. PK catalyzes the last reaction, resulting in net ATP production via anaerobic glycolysis. NADH is used to reduce Hgb-Fe^{3+}.
- The hexose monophosphate shunt (*blue background*; also known as the pentose shunt and the pentose phosphate pathway) generates NADPH that is used to keep GSH in a reduced state in a reaction catalyzed by GR (with cofactor FAD). GSH and NADPH are the major reducing agents in RBCs and are used to maintain Hgb and other proteins in a functional reduced state. G6PD is the rate-limiting enzyme of the shunt. R-5-P, which also is formed from inosine in pig RBCs, can undergo a series of reactions yielding F-6-P and G-3-P for glycolysis (not shown).
- Cb_5R uses FAD and NADH to catalyze the conversion of two molecules of $Fe^{3+}Cb_5$ to two molecules of $Fe^{2+}Cb_5$. The $Fe^{2+}Cb_5$ reduces Hgb-Fe^{3+} to Hgb in a nonenzymatic reaction. Because of the second reaction, Cb_5R is sometimes called methemoglobin reductase (or NADH methemoglobin reductase).

1. When RBCs are incubated with NMB, brilliant cresyl blue, or crystal violet prior to making a blood film, the stain precipitates and stains the RNA and mitochondria to yield punctate or reticulated basophilic structures, hence the term *reticulocyte*.
2. On a Wright-stained blood film, the RNA gives the polychromatophilic RBC its blue or basophilic tinctorial properties. Not all reticulocytes have enough RNA to be detected on a Wright-stained blood film.
3. NMB or other stains (e.g., oxazine, polymethine) are used to detect reticulocytes in some hematology analyzers.

B. The life spans of circulating reticulocytes of nonanemic dogs and people are 1–2 d, after which the cells are mature RBCs. The life spans of cat reticulocytes are more variable. In health, polychromatophilic RBCs are not seen in cattle, horses, llamas, and alpacas because RBC maturation is more complete in the bone marrow.

C. Types of reticulocytes
1. In cats, reticulocytes stained by NMB may be classified by two systems.
   a. The more commonly used system differentiates reticulocytes into either punctate or aggregate forms.[18] The degree of polychromasia seen on a Wright-stained smear correlates with the aggregate RP,[19] as do automated reticulocyte concentrations.
      (1) *Punctate reticulocytes*: RBCs with 2–6 distinct dots of reticulum rather than aggregates (some define differently, e.g., having at least 1 dot or 3–12 dots)
      (2) *Aggregate reticulocytes*: RBCs with aggregates of reticulum rather than only dots
   b. Type I, II, and III reticulocytes[20,21]
      (1) *Type I* (lightly reticulated, oldest form): Cells stain light green with faint blue stippling. Their circulating life span is reportedly 3 d, and the maximum concentration occurs about 10–12 d after the onset of anemia.
      (2) *Type II* (moderately reticulated): Cells stain light green with large dark granules. Their circulating life span is about 12 h, and the maximal concentration occurs about 4 d after the onset of anemia.

Figure 3.5. *continued*

- NADPHD also catalyzes conversion of Hgb-Fe^{3+} to Hgb and thus is another methemoglobin reductase. However, this is a very minor reaction in physiologic states and requires an electron acceptor. Therapeutically, MB can be used as the electron acceptor and is converted to LMB; LMB then reacts nonenzymatically to reduce Hgb-Fe^{3+} to Hgb.
- The diphosphoglycerate (DPG) shunt (*pink background*; also known as the Rapaport–Luebering cycle) provides 2,3-DPG at the expense of ATP production; 2,3-DPG decreases Hgb affinity for O_2 and thus promotes O_2 delivery to tissues. Species vary in the 2,3-DPG content of their RBCs; concentrations are high in dogs, horses, and camelids, but very low in cattle and cats.[14]

1,3-DPG, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; 6-PG, 6-phosphogluconate; ADP, adenosine diphosphate; DPG, diphosphoglycerate; F-1,6-DP, fructose-1,6-diphosphate; F-6-P, fructose-6-phosphate; $Fe^{2+}Cb_5$, ferrocytochrome b_5; $Fe^{3+}Cb_5$, ferricytochrome b_5; G-3-P, glyceraldehyde-3-phosphate; G-6-P, glucose-6-phosphate; GS-SG, glutathione disulfide; Hgb, deoxyhemoglobin; $HgbFe^{3+}$, methemoglobin; $HgbO_2$, oxyhemoglobin; HK, hexokinase; HMP, hexose monophosphate; LMB, leukomethylene blue; MB, methylene blue; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPHD, NADPH dehydrogenase; PEP, phosphoenolpyruvate; PGD, phosphogluconate dehydrogenase; PO_4, phosphate; Prot-SH, protein with reduced sulfhydryl groups; Prot-S-S-Prot, protein with disulfide bridges; R-5-P, ribulose-5-phosphate

(3) *Type III* (heavily reticulated, youngest form): Cells generally are larger than nonreticulated cells and have blue-green cytoplasm and a heavy dark blue granular network. Their circulating life span is about 12 h, and the maximal concentration occurs about 4 d after the onset of anemia.

2. Reticulocyte RNA content occurs on a continuum; there is not always a clear distinction between punctate and aggregate reticulocytes. Although punctate reticulocytes occur in species other than cats (e.g., dogs),[22] they have been differentiated and reported clinically only for cats because they have been considered minor and not of diagnostic value in other species.

D. Reticulocytosis: increased RC, CRP, or polychromasia

1. The degree of increased polychromasia generally correlates with the degree of aggregate reticulocytosis in cats, and with the degree of reticulocytosis in dogs, cattle, and camelids. However, differences may be greater with greater proportions of relatively mature reticulocytes that have enough RNA to be detected by analyzers but not enough to appear polychromatophilic microscopically. Also, cells that appear polychromatophilic with one routine hematology stain may not be detectable with another.
2. In species other than equids, increased RC is generally the best objective evidence of increased erythropoiesis, but the subjective finding of moderately to markedly increased polychromasia is also very supportive. Neither may be present despite a rising Hct once the Hct is only mildly decreased in a resolving anemia. Age should be considered when interpreting reticulocyte values because young animals may have greater reticulocyte reference limits, e.g., dogs in the first few months of life.[23] Also, excitement at the time of blood collection may cause a mild reticulocytosis.[21]
   a. Cattle: After acute blood loss, a reticulocytosis is expected within 3–4 d, and peak production is expected in 7–14 d. A few large (shift) reticulocytes may be seen in the blood before day 3,[24] and the RC may return to baseline while the Hct continues to rise in the later stages of resolution.
   b. Dogs: The response after acute blood loss is not clearly established but is generally reported to mimic the bovine pattern. Some report that the peak response can be seen prior to 7 d.
   c. Cats: The acute removal of RBCs by repeated phlebotomies to create a Hct of 50 % of baseline in five cats resulted in the following:[19]
      (1) The percentage of aggregate reticulocytes increased by day 2, peaked at day 4, and returned to baseline by day 9, even though the cats were still anemic (Hct in the low 20s).
      (2) The percentage of punctate reticulocytes was mildly increased by day 1, peaking days 7 through 16, and the punctate RP did not return to baseline until after the Hct returned to prephlebotomy values (after 3 wk).
   d. Horses: Circulating polychromatophilic RBCs are very rarely seen, e.g., with hemolytic anemia caused by red maple leaf toxicosis or NI.[25] Reticulocytosis has been detected by automated analyzers, but to a limited degree; reticulocyte enumeration is not clinically useful for assessing equine anemias.[26]
   e. Llamas and alpacas: In health, up to about 0.5 % of the RBCs may be reticulocytes. Blood loss or hemolysis causes a detectable reticulocyte response within a few days, peaking 1–2 wk after severe blood loss anemia, but the reticulocytosis may be mild.[27] Serial Hcts can be used to confirm effective regeneration.

E. Reticulocytopenia is a decrease in RC, i.e., less than the lower reference limit. Reticulocytopenia with concurrent persistent anemia is evidence of decreased RBC production. A RC that is WRI also supports decreased RBC production when present with a persistent (at least 3–5 d) moderate or marked anemia.

VII. Blood groups or types

A. General concepts: RBCs can be grouped into different blood groups or types based on the presence or absence of RBC surface antigens, which are sometime called *blood-group factors*. Nearly all blood-group factors are determined by genetics, and each species has different antigens. In people, blood-group factors may function as membrane transporters, receptors, ligands, or structural proteins. A variety of nomenclature systems have been used for blood types.

1. Variations in an RBC surface molecule that is typically expressed in a particular species can create *alloantigens*, antigens recognized as foreign by other individuals of the species, thereby leading to an immune response and the production of *alloantibodies* (antibodies to alloantigens).
2. Although isoantigens and isoantibodies are often considered synonymous with alloantigens and alloantibodies, respectively, the term *isoantigen* is sometimes restricted to epitopes seen as foreign to an individual lacking all variants of a molecule (genetic deficiency) versus epitopes seen as foreign because they are a different form of a molecule (alloantigens). Alloepitopes and isoepitopes can differ on the same molecule.

B. In dogs, at least a dozen blood groups have been described; seven are currently named using the *dog erythrocyte antigen* prefix: *DEA 1, 3, 4, 5, 6, 7,* and *8*. Other antigens recognized by their ability to stimulate alloantibody production are Kai 1, Kai 2, and Dal.

1. DEA 1 is most clinically relevant because it induces acute immune-mediated hemolytic transfusion reactions in DEA 1–negative dogs previously exposed to it. Prevalence varies markedly with breed, but about 60 % of dogs in the United States are DEA 1 positive. Although previously split into subtypes (DEA 1.1, 1.2, and 1.3), there is evidence that DEA 1 is an autosomal dominant trait with at least four alleles yielding dogs with no, low, moderate, or high expression of DEA 1.[28] Although DEA 1 typing results did not align completely with previous DEA 1.2 typing results, the previous DEA 1.2 type was considered "most likely" to reflect DEA 1 positivity.
2. Kai antigens were detected during efforts to develop anti-DEA monoclonal antibodies, and the monoclonal antibodies developed to Kai 1 and Kai 2 are strongly agglutinating. Over 90 % of tested dogs have been Kai 1 positive and Kai 2 negative, with about 5 % negative for both, 1 % positive for only Kai 2, and none positive for both. The clinical significance of anti-Kai alloantibodies in previously sensitized recipients is unknown.
3. Dal expression varies markedly with the breed, being present in most or all greyhounds, golden retrievers, Labrador retrievers, and German shepherds, but absent from many shih-tzus, Doberman pinschers, up to 15 % of Dalmatians, and some individuals of other breeds, including beagles. Dal-negative dogs are at high risk of Dal-incompatible transfusions.
4. Although transfusions of RBCs expressing these blood type alloantigens can induce alloantibody production, so-called naturally occurring (preexisting) alloantibodies that can produce serious transfusion reactions do not exist in dogs. Low titers of naturally occurring weakly reactive anti-DEA 7 alloantibodies have been detected, but data are lacking to know if they impact transfusion efficacy.

5. Clinically apparent acute hemolytic transfusion reactions have been reported only in dogs previously transfused with RBCs positive for DEA 1, DEA 4, Dal, or unknown factors.

C. Cats have an AB blood group system and nonAB feline erythrocyte antigens (FEAs) currently named FEA 1–6 and Mik; Mik might be one of FEA 1–6 (e.g., FEA 1), but this can no longer be tested.[29,30]
   1. The *AB system* has three blood phenotypes: type A, type B, and type AB.
      a. Most cats (70–100 %) are type A, expressing membrane glycolipid and glycoprotein N-glycolylneuraminic acid which is generated from sialic acid N-acetylneuraminic acid by the enzyme cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH).[31] These cats may have weak naturally occurring anti-B alloantibodies.
      b. Blood type B is less common or rare in many breeds but is reported in ~10–35 % of cats in New Zealand and Australia, and in ~25–60 % of Devon rex, Cornish rex, British shorthair, Turkish Van, and Turkish Angora cats. Type B cats have mutations in the CMAH gene causing impaired enzymatic function such that acetylneuraminic acid remains on RBC membranes. These cats have strong naturally occurring anti-A alloantibodies that can cause NI and severe acute hemolytic transfusion reactions.
      c. Type AB is generally rare in the USA (< 1 % of cats), but it is reported in as many as 5–10 % of cats in some countries (e.g., Japan), and it is relatively common in ragdolls (about 20 %). Type AB cats have partial CMAH activity and express glycolylneuraminic acid (A antigen) and acetylneuraminic acid (B antigen). Because of *CMAH* variants, expression of antigen A may be weak in some AB cats.[32] AB cats lack anti-A and anti-B alloantibodies and can receive RBCs from either type A or type B cats; however, plasma from type B cats can cause anti-A transfusion reactions in type AB cats.
   2. FEA 1–6: These feline RBC antigens were discovered via incompatible crossmatches. RBCs expressing FEA 1 appear to be capable of inducing alloantibodies and acute hemolytic transfusion reactions in transfusion-naïve FEA 1–negative cats.[33]
   3. Mik: Mik may belong to the FEA 1–6 group (e.g., FEA 1). Cats are either positive or negative for Mik (named after an alloantibody in a cat named Mike). Mik-negative cats may have naturally occurring anti-Mik alloantibodies that cause acute hemolytic transfusion reactions.[29]

D. Horses: The blood-group systems of horses are relatively complex, with over 30 identified blood factors belonging to at least the seven blood systems that have been recognized by the International Society for Animal Genetics: A, C, D, K, P, Q, and U.[34,35] This yields many thousands of possible RBC types. Blood type components are described by a capital letter to denote the system and a lowercase letter to denote the specific antigen, e.g., Qa, Qb, and Qc. Blood groups may be preceded by "EA" for *erythrocyte antigen*, e.g., EAQ.
   1. The prevalence of some blood factors varies markedly among breeds.
   2. Factors Aa, Ac, Ca, and Qa have particular relevance.
      a. Horses that lack Aa, Ac, or Ca may have naturally occurring anti-Aa, anti-Ac, or anti-Ca antibodies.
      b. Types Aa and Qa are commonly associated with NI; mares may develop anti-Qa or anti-Aa alloantibodies after foaling type Qa or Aa foals.

E. Cattle: The blood-group systems of cattle are very complex, with over 70 known blood-group factors in at least 11 blood systems. With this degree of genetic diversity, it is essentially impossible to find two cattle with the same blood type. However, single transfusions or multiple single-donor transfusions within 5 d have been considered acceptable.[36]

F. Llamas and alpacas: Little information is available regarding blood types in these species. Immunization of rabbits and llamas with RBCs from 12 llamas generated five nonagglutinating hemolytic alloantibodies from the llamas and additional heteroantibodies from the rabbit that recognized six factors designated A through F.[37] However, transfusion of unmatched llama blood appears to carry little risk.[38]

ANALYTICAL PRINCIPLES AND METHODS

I. Complete blood count (CBC): See Appendix A for major concepts pertaining to a CBC.

II. Erythrogram

A. Morphologic evaluation

1. A microscopic evaluation of stained RBCs is an important part of the erythrogram, especially when anemia is present.
2. In the Morphologic Features of RBCs section, the clinical significance of RBCs with abnormal colors, sizes, shapes, inclusions, or other features is described.

B. Hct (synonym, PCV): *hemato-* ("blood") *-crit* (denoting "separation")

1. *Hct* is the percentage of blood volume filled by RBCs and therefore a measure of the O_2-carrying capacity of the blood. If there are 100 mL of blood with a Hct of 45 %, then RBCs occupy 45 mL. Although often referred to as a measure of RBC mass, it is not (note it is not reported in units of mass); the RBC mass is not necessarily reflected by the Hct because changes in fluid volume affect the Hct.
2. Hct accurately reflects the [RBC] in a blood sample if MCV is accurate and WRI. Hct accurately reflects blood [Hgb] if MCHC or CHCM are accurate and WRI. With abnormalities of RBC volume or Hgb content, values for Hct, [Hgb], and [RBC] may not change in parallel.
3. Unit: % (volume %); the SI expression is a unitless decimal fraction, e.g., 0.40 for 40 %, but it is sometimes written as L/L

C. Blood [Hgb]

1. *Blood [Hgb]* is the grams of Hgb per 100 mL of blood. Essentially all Hgb in blood is in RBCs except in a few pathologic states (e.g., intravascular hemolysis causing hemoglobinemia) or after treatment with Hgb-based O_2 carriers (e.g., Oxyglobin).
2. Decreases and increases in [Hgb] reflect anemia and erythrocytosis, respectively, and are not called hypohemoglobinemia or hyperhemoglobinemia.
3. Blood [Hgb] accurately reflects the [RBC] and Hct when the MCHC (or CHCM) and MCV are accurate and WRI, if hemoglobinemia is not present. Blood [Hgb] is a more direct measure of blood's O_2-carrying capacity than is Hct or [RBC].
4. Unit conversion: g/dL × 10 dL/L = g/L (SI unit)

D. [RBC]

1. *[RBC]* is the number of RBCs per unit volume of blood (in clinical jargon, commonly referred to as *RBC count*).

2. The [RBC] accurately reflects Hct and blood [Hgb] if the MCV and MCHC (or CHCM) are accurate and WRI. The [RBC] is used to calculate MCH and can be used to calculate the MCV if the MCV is not directly measured.
3. Unit conversion: (No. × 10^6/μL) × 10^6 μL/L = No. × 10^{12}/L (SI unit)

E. Wintrobe's RBC indices: MCV, MCHC, and MCH
1. *Wintrobe's RBC indices* are three values that are used to characterize RBCs in blood. The MCHC (or CHCM) and MCV are used to classify anemias (see Anemia, Section II.B).
   a. *MCV* is the mean volume per RBC expressed in femtoliters (fL) or cubic micrometers (μm^3).
   b. *MCHC* is the mean cellular Hgb concentration per RBC expressed as grams of Hgb per 100 mL of RBCs (g/dL); g/dL × 10 dL/L = g/L (SI unit). As a concentration, MCHC is affected by the fluid volume within RBCs.
   c. *MCH* is the mean mass of Hgb per RBC expressed in picograms (pg). MCH is not a concentration, so it is not affected by swelling or shrinkage of RBCs.
2. In most blood specimens, not all RBCs are exactly the same (i.e., the RBCs are of different volume, [Hgb], and Hgb mass content). It is important to remember that the MCV, MCHC, and MCH represent the means for all RBCs in the sample. For example, if half the RBCs were large and half were small, the MCV could be WRI.
3. Relationship of indices
   a. Because the MCH represents how much Hgb is in the average RBC, and the MCV represents the volume of the average RBC, the cell Hgb concentration of the average RBC can be calculated by dividing the MCH by the MCV, though this is not the typical calculation that is used (Eq. 3.1a).

$$\text{MCHC} = \frac{\text{MCH}}{\text{MCV}}$$

Example: MCH = 20 pg, MCV = 60 fL **(3.1a)**

$$\text{MCHC} = \frac{20\ \text{pg}}{60\ \text{fL}} = \frac{20 \times 10^{-12}\ \text{g}}{60 \times 10^{-15}\ \text{L}} = \frac{20,000 \times 10^{-15}\ \text{g}}{60 \times 10^{-15}\ \text{L}} = 333\ \text{g/L} = 33.3\ \text{g/dL}$$

$$\text{MCV} = \frac{\text{Hct} \times 10}{[\text{RBC}]} \qquad \text{MCHC} = \frac{\text{blood [Hgb]} \times 100}{\text{Hct}} \qquad \text{MCH} = \frac{\text{blood [Hgb]} \times 10}{[\text{RBC}]}$$

(3.1b)

$$\text{Hct} = \frac{\text{MCV} \times [\text{RBC}]}{10} \qquad \textbf{(3.1c)}$$

   b. When originally described by Wintrobe, the indices represented the calculated interrelationships of three measured values: Hct (reported as a %), blood [Hgb] (reported as g/dL), and [RBC] (reported as 10^6/μL) (Eq. 3.1b).
4. The Wintrobe formulas are used to calculate some of the results generated by impedance and light-scatter cell counters.
   a. Hct is calculated from the measured MCV and [RBC] (Eq. 3.1c). For alpacas and llamas, analyzer Hcts are not reliable.

b. MCHC is calculated from the measured blood [Hgb] and calculated Hct (Eq. 3.1b). For alpacas and llamas, or for samples with unreliable MCV or [RBC], MCHC may be calculated by replacing the instrument Hct with a spun Hct.
c. MCH is calculated from the measured blood [Hgb] and measured [RBC] (Eq. 3.1b). For alpacas and llamas, MCH is typically not calculated or provided because of inaccuracies in measuring [RBC].

5. Relationship of blood [Hgb] and MCHC (with conventional units)
   a. Sample 1: Hct = 50 % and blood [Hgb] = 15 g/dL
      (1) If you have 100 mL of blood with a Hct of 50 % and a blood [Hgb] of 15 g/dL (grams of Hgb per 100 mL of blood), then you have 50 mL of RBCs, 50 mL of plasma, and 15 g of Hgb in the RBCs.
      (2) Therefore, you have 15 g of Hgb per 50 mL of RBCs, or 30 g of Hgb per 100 mL of RBCs, and thus the MCHC = 30 g/dL.
   b. Sample 2: Hct = 33 % and blood [Hgb] = 10 g/dL
      (1) If you have 100 mL of blood with a Hct of 33 % and a blood [Hgb] of 10 g/dL (grams of Hgb per 100 mL of blood), then you have 33 mL of RBCs, 67 mL of plasma, and 10 g of Hgb in the RBCs.
      (2) Therefore, you have 10 g of Hgb per 33 mL of RBCs, or 30 g per 100 mL of RBCs, and thus the MCHC = 30 g/dL.
   c. When the MCHC is 33.3 g/dL, the blood [Hgb] value is one-third of the Hct value (e.g., Hgb = 15 g/dL and Hct = 45 %, or Hgb = 8 g/dL and Hct = 24 %).
   d. Because MCHC values in most noncamelid mammalian blood samples are 32–38 g/dL, the Hgb value typically is about one-third of the Hct value. The MCHC for healthy alpacas and llamas is in the 40s.
6. In addition to calculating the MCHC as described, the ADVIA analyzers measure the [Hgb] of each individual RBC by analysis of light scatter, which is related to cell [Hgb] in isovolumetrically sphered RBCs. This provides the following additional erythrogram results for the ADVIA:
   a. *CHCM* is the mean of the cellular Hgb concentration measured in each RBC, with the same concentration and units as the MCHC but determined directly by light scatter. It is unaffected by many of the spectral interferences that can cause a falsely increased MCHC. It is not valid for camelids.
   b. *CH* is the mean Hgb content (mass) of RBCs, as is MCH, but it is the mean Hgb content of individual cells as calculated from direct measurements of each cell's volume and Hgb concentration (the mean of the CH histogram).
   c. *HDW* reflects the amount of variation in the RBC Hgb concentration. It is the SD (g/dL) of the RBC Hgb concentrations in the cell Hgb concentration histogram used to determine CHCM. Increased HDW is called *anisochromasia*.

F. RDW
1. *RDW* is a calculated value that reflects the amount of variation in RBC volumes (see Morphologic Features of RBCs, Section VI). Increased RDW is an indication of anisocytosis, but visual smear anisocytosis may be present without an increased RDW. A decreased RDW is not of clinical significance.
2. The shape of camelid RBCs prevents accurate RBC volume measurement, so a valid RDW is not generated for alpacas and llamas.
3. RDW is determined in multiple ways, but for each method, the "width" in RDW refers to the width the RBC volume distribution, not the width of RBCs.

a. *RDW-CV* (and traditional RDW) is the coefficient of variation (CV) of the RBC volumes that are used to determine the MCV: RDW-CV = 100 × (SD of MCV ÷ MCV). Equivalently, it can be calculated from the difference in RBC volumes 1 SD above and 1 SD below the mean.[39]
   (1) RDW-CV is therefore influenced mostly by the central 68.26 % of the cells (± 1 SD of the mean), so it may not adequately reflect anisocytosis caused by populations of large or small RBCs when most RBCs have a typical distribution (i.e., when left or right shoulders alter a typical volume distribution histogram peak).
   (2) As a ratio, RDW-CV is affected by the magnitude of the MCV: The same distribution width yields a greater value when there is a microcytosis and a lesser value when there is a macrocytosis. Thus, RDW-CV overestimates anisocytosis in microcytic states and underestimates it in macrocytic states. RDW-CV remains WRI if MCV and its SD change proportionately.

b. *RDW-SD* reflects the size distribution of RBC volumes used to determine the MCV, but it is not a SD (standard deviation). It is the difference between high and low volumes at a given frequency level above baseline (20 % for Sysmex or ProCyte Dx instruments) of the volume distribution histogram, i.e., the width of the peak at that level. This typically includes more RBCs than are present in the SD (standard deviation) used for RDW-CV calculation. Assessment is not done over the entire baseline breadth of the histogram because that may include small RBC agglutinates (doublets, triplets) or the coincidental analysis of RBCs that happened to pass through the laser together (RBC coincidence), platelet clumps, giant platelets, and artifacts including electrical interference. However, low shoulders of macrocytic or microcytic RBCs are not included. RDW-SD may not be reported with some abnormal distributions, e.g., some bimodal distributions.

c. Examination of the volume frequency distribution histogram provides more information than an RDW value alone.

4. Units: RDW-CV is reported in %; RDW-SD is reported in fL.

G. nRBCs

1. nRBCs seen during examination of a blood film are enumerated by counting the number of nRBCs seen while 100 (or more) WBCs are differentiated and counted. The conventional method of recording the number of nRBCs is in units of nRBC/100WBC (e.g., 10 nRBC/100WBC). This method is useful for correcting a measured [WBC] but may give a false impression of the number of nRBCs in blood. For example, two blood samples each have 50 nRBCs/100WBCs. If one sample has a [WBC] of 50,000/μL and the second sample has a [WBC] of 500/μL, then the [nRBC] in the first sample is 100 times as great as the [nRBC] in the second sample (25,000/μL vs 250/μL). Therefore, reporting the [nRBC] instead of using the conventional method provides a more consistent indication of the degree of rubricytosis.

2. Microscopic (hemocytometer) and most electronic methods determine the [WBC] by counting nucleated cells (WBCs or nRBCs) or their nuclei. By these methods, a *measured (instrument) [WBC]* represents the sum of nucleated cell concentrations (WBCs plus nRBCs). Correction of the measured [WBC] is necessary to obtain the actual [WBC], the accuracy of which is limited by inaccuracies in nRBC enumeration. Alternatively, and much less commonly, a nucleated cell concentration, not a [WBC], is reported along with percentages and concentrations for nRBCs and each type of WBC.

3. A [nRBC] can be calculated from a measured [WBC] or a corrected [WBC].
   a. The most direct method is shown in Eq. 3.2a.

$$[\text{nRBC}] = \text{measured [WBC]} \times \frac{\#\text{ nRBC per 100 WBC}}{100 + (\#\text{ nRBC per 100 WBC})}$$

Example: measured [WBC] = 20,000/μL, nRBC = 25/100 WBC (**3.2a**)

$$[\text{nRBC}] = 20{,}000/\mu\text{L} \times \frac{25}{125} = 4000/\mu\text{L}$$

$$[\text{nRBC}] = \text{corrected [WBC]} \times \frac{\#\text{ nRBC}}{100\text{ WBC}}$$

Example: corrected [WBC] = 16,000/μL, nRBC = 25/100 WBC or $\frac{25\text{ nRBC}}{100\text{ WBC}}$ (**3.2b**)

$$[\text{nRBC}] = \frac{16{,}000\text{ WBC}}{\mu\text{L}} \times \frac{25\text{ nRBC}}{100\text{ WBC}} = \frac{4000\text{ nRBC}}{\mu\text{L}} = 4000/\mu\text{L}$$

$$[\text{nRBC}] = \text{measured [WBC]} - \text{corrected [WBC]} \quad (\textbf{3.2c})$$

   b. A [nRBC] can be calculated from the corrected [WBC] by using Eq. 3.2b.
   c. If the corrected [WBC] and measured [WBC] are known, the [nRBC] is easily calculated by using Eq. 3.2c.
4. Microscopic determination of [nRBC] followed by correction of WBC data is of diagnostic value but not very precise. Automated measurement of [nRBC], now available with Sysmex XN analyzers, provides more precise nRBC measurements based on forward scatter assessment of volume and side fluorescence assessment of nucleic acid content, but results with canine and feline samples can be inaccurate for several reasons.[40]

H. Reticulocytes
1. Enumeration of blood reticulocytes is an important part of an erythrogram, especially in anemic dogs, cats, and cattle.
2. These are the three common methods of enumerating blood reticulocytes:
   a. The *reticulocyte concentration* (RC) is the concentration of reticulocytes in blood expressed as the number of reticulocytes per μL (or L) of blood. The RC is generally the preferred method for evaluating marrow response to anemia. In clinical jargon, the RC is often called the reticulocyte count, but a reticulocyte count is used to calculate a RC.
   b. The *reticulocyte percentage* (RP) is the percentage of RBCs that are reticulocytes in a blood sample, e.g., if there are 10 reticulocytes per 1000 RBCs, then the RP is 1.0 %. In clinical jargon, the RP is also often called the reticulocyte count.
   c. The *corrected reticulocyte percentage* (CRP) is a calculated percentage that represents the RP if the animal were not anemic (see the following Section III.K).

III. Methods
A. Many analyzers and methods are available for generating CBCs in clinics and referral laboratories, and new ones continue to be developed. To limit errors, all require appropriate maintenance, use, and knowledge of potential preanalytical, analytical, and postanalytical factors that can negatively affect results. None can replace microscopic examination of blood smears by a trained specialist. Representative examples of methods are provided here.

B. The elliptical shape, small size, and high concentration of camelid RBCs yields inaccurate results for [RBC], MCV, and therefore most quantitative RBC data generated by hematology analyzers. Camelid MCV may be calculated from spun Hct and a valid [RBC]: MCV = (spun Hct × 10)/[RBC], but measurement of an accurate [RBC] is problematic. MCHC may be calculated from spun Hct and [Hgb]: MCHC = ([Hgb] × 100)/spun Hct. Measurement of Hct by conductivity may also be problematic; compared to spun Hct, Hct values from a blood gas and electrolyte analyzer were almost one-third lower for samples from healthy alpacas and llamas.[41]

C. Microhematocrit

1. One of the easiest, most accurate and most reproducible methods of assessing [RBC] in blood is by the centrifuged microhematocrit. Use of the microhematocrit also enables visual subjective evaluation of plasma for hemolysis, lipemia, and icterus. It is needed for camelid blood because of inaccuracies in automated analysis caused by small elliptocytic RBCs.
2. When first described, the term *hematocrit* (Hct) was the name of the procedure used to separate blood into its major components: packed RBCs, buffy coat layer, and plasma. With common use of the procedure, Hct started to indicate the major result of the procedure (i.e., Hct, PCV). When a small capillary tube was introduced for the procedure, it became the *microhematocrit* method to differentiate it from the larger Wintrobe Hct tubes. Today, the terms *hematocrit* and *packed cell volume* are frequently used as synonyms. However, some people differentiate the centrifuged microhematocrit from the calculated Hct by referring to the former as the PCV or spun Hct and to the latter as the Hct, calculated Hct, or analyzer Hct.
3. High-speed centrifugation of blood in a microhematocrit tube separates the blood cells into layers based on the density of cells. The buffy coat layer contains platelets, lymphocytes, monocytes, eosinophils, neutrophils, basophils, and nRBCs (listed from least to most dense). The buffy coat cells typically occupy about 0.5–1.0 % of the blood volume. The RBCs are below the buffy coat.
4. The appropriate time (usually 2–10 min) and force used (usually about 10,000–16,000 × g, which should be reached within 30 s) vary with the centrifuge, shorter times being used with greater forces. This can be checked by making duplicate Hct measurements of fresh samples, at least one with a Hct > 50 %, beginning at 2 min and increasing by 30 s intervals until the values are unchanged over two consecutive time intervals. The second of the two times yielding similar results is the minimum centrifugation time.
5. Based on studies of human blood, when centrifugation is adequate, only ~1–3 % of a blood sample's plasma is trapped in the RBC layer.[42] This falsely increases the spun Hct by up to ~2 (usually < 1) percentage points, e.g., from 25 % to at most 27 %. The presence of many RBCs that pack less tightly (e.g., spherocytes) may increase the percentage of trapped plasma, but the increase appears to be slight.
6. Because of potential inaccuracies in measuring the [RBC] and MCV, which are used for the calculated Hct, the microhematocrit tube method (spun) is generally considered the gold standard for determining blood Hct. However, erroneous microhematocrit results can be produced by the following:
   a. Inadequate mixing of blood before the microhematocrit tubes are filled could produce either a falsely high or a falsely low Hct.
   b. With an inadequate volume of blood in the EDTA tube, the hypertonic salt shrinks the RBCs and thus gives a falsely low Hct.

c. Inadequate centrifugation (insufficient duration of high-enough centrifugal force) may cause a falsely high Hct because plasma remains within the loosely packed RBCs. Optimal equipment maintenance includes regular checks for accuracy and reproducibility of the timer (with a stopwatch) and centrifugal speed (with a tachometer).
d. Misreading of the Hct, including misidentifying the interface between RBCs and buffy coat, may result in erroneous values.
e. Microhematocrit tube material and bore size can affect Hct, but significant differences are not expected with commercial products.

D. Impedance methods
1. The basic principles of impedance counters are described in Analytical Principles and Methods, Section II.B, in Chapter 2. Most impedance counters measure MCV, blood [Hgb], and [RBC]. From those measurements, most instruments calculate Hct, MCHC, MCH, and RDW (Eq. 3.1 for the Wintrobe equations).
2. For most samples, a properly adjusted and calibrated impedance cell counter can provide reliable data if clots and platelet clumps are not present.[43]
a. For canine blood, most counters can typically differentiate RBCs and platelets, and thus can provide reliable RBC and platelet data. However, numerous large platelets or small RBCs (e.g., in Fe deficiency) can result in false data.
b. For feline blood, most counters cannot reliably differentiate RBCs and platelets because of the overlap in cell volumes. Because the instrument classifies large platelets as RBCs, the [platelet] is falsely low. Because of relative numbers, the degree of inaccuracy in the RBC data is typically low except when there is a marked anemia.
c. For equine blood, most counters can typically differentiate RBCs and platelets, and thus can provide reliable RBC and platelet data. The major exception is when the MCV is relatively low and approaches 30 fL (seen primarily in foals).
d. For bovine blood, most counters can typically differentiate RBCs and platelets, and can provide reliable RBC data.
e. For alpaca and llama blood, high [RBC], small RBC volume, and elliptical shape of RBCs may pose problems for values other than [Hgb], making the spun Hct particularly important. If the [RBC] is accurate, it may be used with the spun Hct to calculate MCV.

E. Optical or laser flow cell methods (e.g., CELL-DYN, Bayer Technicon H1, Siemens ADVIA, Sysmex, ProCyte)
1. For basic principles, see Chapter 2, especially Figure 2.5. The flow cytometric aspects of the CELL-DYN are used in addition to impedance to evaluate WBCs, whereas the ADVIA uses only flow cytometric methods to evaluate WBCs, RBCs, and platelets. Sysmex and ProCyte Dx analyzers combine flow cytometry, impedance, and optical fluorescence methods.
2. The ADVIA uses unique optical methods of evaluating RBCs and reticulocytes. With a special diluent, RBCs are isovolumetrically sphered before they pass through a laser beam in which they scatter light. Sphered cells scatter light more predictably than biconcave discs. Not all RBCs are sphered, e.g., ovalocytes of camelids, for which the analyzer cannot generate accurate volume and Hgb concentration data. However, when using the equine instrument settings, some have considered the errors in [RBC] and [Hgb] with alpaca samples small enough to report and use for calculation of Wintrobe indices, at least if severe anemia and microcytosis are not present.[44]

a. As a RBC or platelet passes through the laser beam, its presence is detected by light scatter. The low-angle light scatter (2–3°) is used to determine a cell's volume, whereas high-angle light scatter (5–15°) is used to determine the cell's refractive index. The refractive index is used to differentiate RBCs and platelets (platelets refract less than RBCs) and to determine the Hgb concentration (HC) in each individual RBC (a greater RBC HC causes a greater refraction of light).
b. From the data gathered as each RBC passes through the flow cell, the following RBC features are measured or calculated:
   (1) *V* is the volume of an individual RBC.
   (2) *MCV* is the mean of the individually measured RBC volumes.
   (3) *RDW* is the CV of individual RBC volumes.
   (4) *[RBC]* is the number of RBCs detected in a given blood volume.
   (5) *Hct* is the percentage of blood volume occupied by RBCs as determined from MCV and [RBC] data (Eq. 3.1c).
   (6) *HC* is the Hgb concentration in an individual RBC.
   (7) *CHCM* is the mean Hgb concentration in RBCs (mean of HC values).
   (8) *HDW* is the SD of HC values.
   (9) *CH* is the mean amount (mass) of Hgb in RBCs. The amount of Hgb in individual RBCs is calculated from each cell's HC and V, i.e., pg/fL × fL = pg.
c. Data from RBC analysis can be graphically displayed by the instrument. For example, a RBC cytogram is constructed from the volumes (V) and the [Hgb] in individual RBCs (HC) (Figure 3.6). The displayed data provide a visual representation of the morphologic classification of RBCs (see Anemia, Section II.B) and can be useful for recognizing subpopulations that may not be large enough to cause increased or decreased MCH, MCHC, or CHCM (mean values).
d. A dye (oxazine 750) that stains cellular RNA is used to differentiate reticulocytes from mature RBCs by absorbance spectroscopy. The dye interacts with RNA in reticulocytes, which are then identified by their increased light absorption. With this differentiation, the instrument determines mean Hgb content (mass) and mean cell volumes of mature RBCs and reticulocytes separately. The reticulocyte features generated by the ADVIA include the following:
   (1) *#RETIC*: number of reticulocytes in a given volume (same as RC)
   (2) *MCVr*: mean of the individually measured reticulocyte volumes
   (3) *CHCMr*: mean Hgb concentration in reticulocytes
   (4) *RDWr*: coefficient of variation of individual reticulocyte volumes
   (5) *HDWr*: SD of Hgb concentrations in individual reticulocytes
   (6) *CHr*: mean mass (amount, content) of Hgb in reticulocytes, calculated as MCVr × CHCMr/100
   (7) *CHDWr*: SD of reticulocyte Hgb mass as displayed in a CHr histogram (values used for calculating CHr)
   (8) Percentages of reticulocytes considered to be microcytic, macrocytic, hypochromic, hyperchromic, or with a low or a high Hgb content
   (9) Differences (delta values) between mature and reticulocyte MCV, CHCM, CH, CHDW, RDW, and HDW
e. Like the other instruments, the ADVIA lyses RBCs and uses a cyanide-free reagent (or cyanide) method to determine the total blood [Hgb]. With this blood [Hgb] and other data, it calculates the following:

(1) MCHC using the blood [Hgb] and the blood Hct (Eq. 3.1b): The MCHC should agree with the CHCM if the measured values are accurate and free Hgb is not in the blood's plasma.
(2) MCH using the blood [Hgb] and the blood [RBC] (Eq. 3.1b): The MCH should agree with the CH if the measured values are accurate and free Hgb is not in the blood's plasma.

f. *Calculated [Hgb]*, the [Hgb] (g/dL) attributable to Hgb that was within RBCs (not in plasma), is calculated as Hct × CHCM/100 (compare to the calculation for MCHC in Eq. 3.1b).

g. Hgb_{delta} is the difference (in g/dL) between the spectrophotometrically measured sample [Hgb] and the calculated [Hgb]. If Hgb_{delta} is increased, it indicates the presence of plasma Hgb (hemolysis), the presence of a Hgb-based O_2 carrier (e.g., Oxyglobin) in the plasma, or an interference such as occurs when many RBCs contain Heinz bodies that cause a greater false increase in [Hgb] than in CHCM.[45]

3. Sysmex and ProCyte Dx analyzers use impedance for [RBC], Hct, MCV, and related values, optical fluorescence for reticulocyte assessment, and a sodium lauryl sulfate photometric method for [Hgb]. MCH and MCHC are calculated routinely, and RDW is reported as both RDW-SD and RDW-CV. Reticulocytes are assessed by fluorescence flow cytometry after a fluorescent dye (polymethine) binds to cytoplasmic nucleic acids, and the reticulocyte percentage (RET%), reticulocyte concentration (RET#), immature reticulocyte fraction (IRF), and reticulocyte Hgb content (RET-He) are reported. Note: The ProCyte One detects reticulocytes by altered light scatter caused by increased internal complexity after RNA is precipitated and stained by NMB.

a. *IRF* is the percentage of reticulocytes that are considered immature; it is the sum of the percentage of medium-fluorescence reticulocytes (*MFR*) and the percentage of high-fluorescence reticulocytes (*HFR*), the more mature low-fluorescence reticulocytes not being included. Fluorescence increases with the amount of RNA in a reticulocyte, and therefore with immaturity.

b. *RET-He*, the reticulocyte Hgb equivalent, is similar to CHr and reflects the average mass (pg) of Hgb in reticulocytes. RET-He is calculated by a proprietary log transformation equation based on the curvilinear relationship between mean mature RBC forward light scatter (arbitrary units) and MCH.[46] This relationship is applied to the mean forward scatter of the reticulocyte population to yield the RET-He for canine samples tested on ProCyte Dx analyzers.[47] Because forward scatter is affected by RBC volume irrespective of RBC Hgb content, RET-He may be falsely increased by macrocytosis or falsely decreased by microcytosis.[47]

F. Quantitative buffy coat (QBC) analysis (IDEXX VetAutoread)

1. Major concepts that pertain to the QBC analysis are described in Analytical Principles and Methods, Section II.B.6, in Chapter 2. Erythrogram data generated by the QBC analyzer include the Hct, blood [Hgb], MCHC, and RP for dogs and cats. The presence of nRBCs may be noted in some samples.
2. The QBC Hct is obtained by centrifugation, and the result should agree with the result obtained by the microhematocrit method.
3. The QBC MCHC is an estimate and is determined by the relationship between the MCHC and the density of the RBCs. The lower the MCHC, the less dense the RBCs.

a. The analyzer measures the RBC density by determining the depth of a plastic float in the RBC layer. A lower RBC density allows the float to penetrate further into the RBC layer. Once the density is determined, the instrument calculates a QBC MCHC.

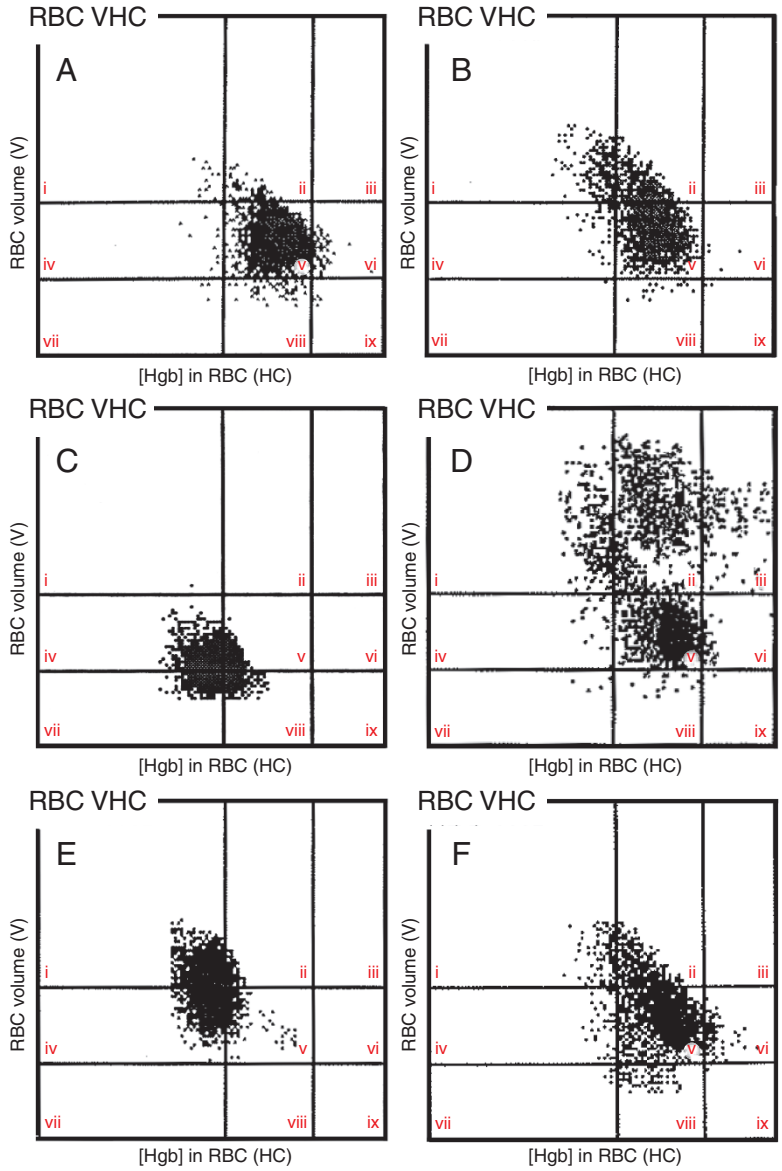


Figure 3.6. RBC VHC (volume hemoglobin concentration) cytograms from dogs. With ADVIA analyzers, the volume (V) and Hgb concentration (HC) of each RBC is graphically displayed in a RBC VHC cytogram often called the tic-tac-toe scatterplot. In health, most RBCs are within region v of the cytogram, indicating they are normochromic normocytes. Macrocytes are displayed in regions *i*, *ii*, and *iii*; microcytes are in regions *vii*, *viii*, and *ix*. Hypochromic RBCs are in regions *i*, *iv*, and *vii*; hyperchromic RBCs are in regions *iii*, *vi*, and *ix*. Shifts in the RBC distribution represent changes in RBC populations, some of which may be detectable before enough RBCs are altered to create increased or decreased MCV or MCHC (CHCM). It is helpful to be able to interpret graphical data generated by a hematology analyzer.

A. Health: There are no RBC population shifts from the central normochromic normocyte region (region *v*). RBC indices from this nonanemic dog were WRIs. A dog with a normocytic normochromic nonregenerative anemia would typically have a similar pattern.

b. Platelet clumps or blood clots in the sample can accumulate above or below the plastic float and thus alter the depth of the float in the RBC layer. In such samples, the QBC MCHC could be either falsely decreased (if platelets or clots pushed the float down into the RBCs) or falsely increased (if platelets or clots prevented the float from sinking).

4. The QBC blood [Hgb] is calculated from the QBC Hct and the QBC MCHC by using a rearranged Wintrobe index formula (Eq. 3.1b, second equation).
   a. If the QBC MCHC is falsely decreased, the QBC blood [Hgb] is falsely decreased.
   b. If the QBC Hct is falsely decreased, the QBC blood [Hgb] is falsely decreased.
5. RP is based on increased RNA fluorescence at the top of the RBC layer, and the presence of nRBCs is suggested by increasing DNA fluorescence toward the top of the RBC layer.

G. [RBC] by the hemocytometer method

1. A hemocytometer can be used to measure [RBC] in blood. Blood is diluted (usually in a commercial kit for diluting and staining cells), and then the diluted blood is used to charge the hemocytometer chamber (Figure 2.3). A microscope is used to enumerate the RBCs within certain grid areas in a standardized fashion, and then a [RBC] is calculated using a formula that adjusts for sample dilution and volume within the counted grid areas.

Figure 3.6. *continued*

B. Regenerative anemia (Hct = 24 % and reticulocytes = 231 × 10^3/μL): A population of RBCs extends into region *i*. This pattern is typical of regeneration because immature RBCs are hypochromic macrocytes. This dog had an increased MCV, decreased MCHC and CHCM, and increased RDW.

C. Fe-deficiency anemia (Hct = 27 %, hypoproteinemia): The RBCs have shifted from region *v* into regions *iv*, *vii*, and *viii*, reflecting mostly hypochromic and/or microcytic RBCs. The MCV, MCH, MCHC, and CHCM were all decreased. With early Fe-deficiency anemia, many RBCs may be in region *v*, with only a subpopulation in regions *iv*, *vii*, or *viii*.

D. IMHA with spherocytosis, agglutination, and regeneration (spun Hct = 22 %): RBCs extending into region *i* from the main population in region *v* reflect an increase in polychromatophilic RBCs (regeneration as in cytogram B). In addition, a cloud of small RBC agglutinates appears in region *ii,* falsely increasing the MCV and falsely suggesting the presence of normochromic macrocytes; larger agglutinates are excluded from analysis. Concurrent hemolysis falsely increased the MCH and MCHC in this dog, the latter falsely suggesting a hyperchromic anemia (MCHC = 44 g/dL) when it was normochromic based on CHCM (35 g/dL) and VHC distribution (relatively few cells appear in regions *iii*, *vi*, or *ix*). Although this dog had many spherocytes, which appear microcytic and hyperchromic microscopically, there is no population in region *ix* because spherocytes are generally normocytic normochromic cells (region *v*) and only look small and dark because they are balled up on the slide.

E. 6-d-old blood sample: RBCs have largely shifted from region *v* into regions *i* and *iv* because of in vitro cell swelling which made the RBCs larger and diluted their Hgb content, thus decreasing MCHC and CHCM (but without affecting MCH). The RBC indices indicated macrocytic (MCV = 90 fL) hypochromic (CHCM = 27 g/dL) RBCs in this nonanemic dog. The blood sample was delayed in transit. Overnight mailing is recommended, but less severe RBC shifts that create inaccurate RBC indices occur by 24 h and even within several hours.

F. PIMA plus severe collagen myelofibrosis (spun Hct = 18 %): The population of RBCs extending into region *i* mimics a regenerative anemia (frame B), but this dog had macrocytic normochromic RBCs with ovalocytosis and without regeneration (reticulocytes = 18,100/μL). This constellation of findings is predictive of PIMA with myelofibrosis. The similarity of patterns B and F emphasize the importance of assessing RC and a blood smear.

2. Determination of the [RBC] by the hemocytometer method is typically imprecise and thus is not recommended for routine assessment of [RBC].

H. Hct and blood [Hgb] determined by conductivity
1. Some electrochemical instruments that are designed to measure blood gas analytes or electrolytes also generate Hct and blood [Hgb] as part of their profile results.
2. The Hct is determined by measuring the electrical conductivity of the blood and then converting that value to a Hct based on an inverse relationship between conductivity and blood cell concentration; electrical conductivity of blood decreases with greater blood cell concentration. Because the [RBC] is typically much greater than the [WBC] or [platelet], a blood sample's conductivity depends primarily on the [RBC]. Because [RBC] and Hct are strongly correlated, a blood sample's conductivity changes inversely with its Hct. Some analyzers correct for changes in electrolyte concentrations.
3. For some analyzers, the Hct that is derived via conductivity is converted to a blood [Hgb] by using Wintrobe's MCHC formula (Eq. 3.1b, second equation) and an assumed MCHC (e.g., 33 g/dL). If the blood sample's MCHC is not 33 g/dL, then the conversion yields an incorrect blood [Hgb]. Because [Hgb] is used in the formula to calculate the base excess of blood, the base excess may also be in error.
4. Other instruments (e.g., Nova pHOx) determine the Hct by conductivity and determine the blood [Hgb] by adjusting the conductivity data based on a photometric measurement of the percentage of Hgb saturation with O_2 (sO_2).
5. The Hct and blood [Hgb] derived by conductivity should be considered estimates. Any change in the blood that alters its conductivity alters the derived Hct and blood [Hgb], e.g., marked leukocytosis yields a falsely increased Hct, and marked hypoproteinemia yields a falsely decreased Hct.

I. Blood [Hgb]
1. Blood [Hgb] is measured by several methods. In general, a lysing solution releases Hgb from RBCs, and the Hgb undergoes oxidation and other reactions to form a heme product that is detected spectrophotometrically with minimal interference from remnant cell material. [Hgb] was measured by cyanomethemoglobin methods for many years, but because of concerns about working with or disposing of minute amounts of cyanide, cyanide-free methods have been developed. High concentrations of cells or the presence of particles that interfere with light transmission may result in falsely increased absorbance and therefore falsely increased blood [Hgb] (see Anemia, Section II.B.12).
2. Portable hemoglobinometers have been used for many decades for measuring blood [Hgb].
   a. The HemoCue AB determines the blood [Hgb] by measuring the absorbance of azide metHgb at two wavelengths. Evaluation at two wavelengths may decrease the false increases caused by spectral interference. It has been shown to have good agreement with blood [Hgb] measured by cyanmethemoglobin spectrophotometry.[48]
   b. A so-called Hct meter uses reflectance photometry to measure [Hgb] from a spot of whole blood applied to a test strip; the estimated Hct is calculated from the [Hgb], and both are reported. When compared to a reference analyzer (ADVIA), negative or positive Hct differences of ≥ 5 percentage points were not uncommon.[49]
3. The LaserCyte Hematology Analyzer measures blood [Hgb] by transmittance of light from two light-emitting diodes, one for methylene blue–stained whole blood and one for free Hgb after RBC hemolysis by a hypotonic solution with surfactant. Comparison of absorptions between the two methods is used to assess result validity.

Table 3.3. Potential effects of selected sample and patient conditions on Hct and values used to calculate Hct

Condition	Spun Hct[a]	Calc. Hct[b]	MCV[c]	[RBC]	Cond. Hct[d]
Inadequate mixing	↓ or ↑	↓ or ↑	—[e]	↓ or ↑	↓ or ↑
Short sample; RBCs shrink	↓	—[f]	↑[g]	—	↓
Clots in sample	↓ or ↑	↓	—	↓	↓
In vitro hemolysis	↓	↓	—	↓	↓
Inadequate centrifugation	↑	NA[h]	NA	NA	NA
Agglutination	—	↓	↑	↓	?[i]
Very small RBCs	—	↓ likely	↑	↓	—
Hyponatremia	—	↓	↓	—	↑
Hypernatremia	—	↑	↑	—	↓
Hyperglycemia (marked)	—	↑	↑	—	—
Leukocytosis	—	—	—	—	↑
Thrombocytosis (marked)	—	(↑)	(↓)	(↑)	↑
Hyperproteinemia (marked)	—	—	—	—	↑
Hypoproteinemia	—	—	—	—	↓

[a] Spun Hct is the Hct determined by centrifugation, either the microhematocrit method or the QBC method.
[b] Calc. Hct is the Hct calculated by impedance or optical cell counters after measuring MCV and [RBC].
[c] MCV measured by impedance or light scatter
[d] Cond. Hct is the Hct determined by electrochemical instruments that measure the conductivity of blood.
[e] —, no effect
[f] Little or no effect because RBCs rehydrate in analyzer buffer (anticoagulant dilution factor is insignificant)
[g] This is a potential effect with excess K_2EDTA and Technicon analyzers, but not with most other instruments (see the text).
[h] NA, not applicable
[i] Not found, but Hct may be affected by RBC settling, and RBC agglutination may promote settling

J. Errors and discrepancies in RBC data determined by centrifugation, impedance, optical, or conductivity methods can occur for a number of reasons (Table 3.3). When repeatable discrepancies of greater than three Hct percentage points occur between appropriately generated spun Hct and analyzer methods for a particular sample, the sample's spun Hct is likely the more accurate result. If such differences occur consistently, they are likely related to analyzer calibration or other analytical factors. If they occur sporadically, the following should be considered.
   1. Inadequate mixing: Inadequate mixing of blood prior to filling a microhematocrit tube can cause an erroneous Hct. RBCs tend to settle if the anticoagulated blood is left standing in a tube rack, especially with pronounced rouleaux, including in healthy horses.
      a. Filling the microhematocrit tube with the top portion of that blood yields a falsely decreased Hct by any method, whereas filling with the bottom portion yields a falsely increased Hct.
      b. Hct values may differ between methods because the blood was properly mixed prior to one assay but not the other.

2. Inadequate sample volume: Collecting or transferring an inadequate amount of blood into an EDTA tube (e.g., 0.5 mL of blood into a tube designed for 3 mL of blood) results in a relative excess amount of EDTA solution. The excess EDTA solution creates hypertonic plasma and RBCs shrink because of osmosis; the smaller RBCs occupy less volume, and thus a spun Hct is falsely decreased. However, when these RBCs are suspended in what is normally an isotonic diluent prior to impedance counting, they quickly gain volume, and thus the MCV and calculated Hct may not be affected. The conductivity Hct may be falsely decreased because the greater concentration of cations and anions from the anticoagulant increases conductivity.
3. Clots: The presence of small clots can have adverse effects on all methods of determining Hct. The clotting removes RBCs from suspension, and thus the Hct on the remaining nonclotted blood is falsely decreased. However, very small clots could enter the microhematocrit tube and result in incomplete RBC packing and thus falsely increase spun Hct. The effects of clots are less predictable with impedance and conductivity methods because the clots may not allow proper sampling by the instrument or may plug the small orifices or tubing; no result may be reported.
4. In vitro hemolysis: All three Hct methods yield falsely decreased Hct if RBCs were lysed during blood collection or handling; the Hct may be correct for the sample, but the sample is not representative of the animal's blood. Mild hemolysis would have a minimal effect, but gross hemolysis would create larger errors.
5. Inadequate centrifugation: A spun Hct is falsely increased when centrifugation of the microhematocrit tube is inadequate because of an inadequate g-force or inadequate time of centrifugation. The spun Hct includes a very small amount of trapped plasma (< 3 % error, which would increase a Hct of 40 % by no more than 1.2 percentage points to as much as 41 %), but poor centrifugation can leave more plasma mixed with the packed RBCs.
6. RBC agglutination: The presence of agglutinated RBCs can falsely increase the MCV and falsely decrease the [RBC] determined by impedance or optical methods because of two factors: (i) very small clusters of RBCs are counted as one large RBC, and (ii) large clusters of RBCs are too large to be identified as RBCs and are excluded from the counting. Consequently, the Hct calculated from these values is unreliable. Effects vary depending on the sizes of the RBC clusters and instruments' size limits for exclusion (the falsely decreased [RBC] times a falsely increased MCV may yield a nearly correct calculated Hct).
7. Small RBCs: Some RBCs of animals (e.g., sheep, goats, young horses, and camelids) may be too small to be recognized as RBCs by impedance or light scatter. If the smaller RBCs are not included in the MCV determination, the instrument generates a falsely increased MCV. Concurrently, the [RBC] would be falsely decreased and the [platelet] may be falsely increased. Because the calculated Hct is the product of the MCV and [RBC], the effect on calculated Hct varies depending on the relative errors for MCV and [RBC], but a false decrease is likely. Spun Hct may be falsely increased if centrifugation times are not long enough to sufficiently pack the small RBCs.
8. Abnormal plasma osmolality (tonicity)
    a. When an animal has a persistent and marked decrease in plasma osmolality because of hyponatremia and hypochloremia, the osmolality within RBCs also decreases. When those RBCs are placed in an isotonic diluent prior to counting, the RBCs shrink as H_2O moves from the cells via osmosis.[50] Thus, the measured MCV is falsely decreased, resulting in a falsely decreased calculated Hct. The opposite

osmosis occurs when an animal has a persistent and marked plasma hypernatremia and hyperchloremia and thus the MCV and calculated Hct are falsely increased.[50] The conductivity Hct may also be affected by marked changes in plasma [Na^+] and [Cl^-] if analyzers do not correct for them; decreased conductivity caused by hyponatremia and hypochloremia would falsely increase the Hct.

b. With marked hyperglycemia (e.g., hyperosmolar syndrome), MCV and calculated Hct may be falsely increased from in vivo glucose diffusion into RBCs such that when tested, RBCs draw in H_2O osmotically from the analyzer buffer as for hypernatremia.

9. Marked leukocytosis, marked thrombocytosis, or marked hyperproteinemia: Each can reduce the conductivity of blood and thus cause a falsely increased conductivity Hct. If the impedance cell counter cannot differentiate platelets from RBCs and thrombocytosis is marked, the measured [RBC] will be falsely increased and probably the MCV will be falsely decreased. If the error in the [RBC] is greater than the MCV error, then the calculated Hct will be falsely increased.
10. Marked hypoproteinemia: This increases the conductivity of blood, and thus the conductivity Hct is falsely decreased.
11. A falsely decreased MCV may occur when an electronic cell counter cannot differentiate large platelets from RBCs. This is primarily a problem when there is a marked thrombocytosis and a concurrent marked anemia. Such erroneous results can be suspected or recognized by other findings, including microscopic examination of the blood that does not confirm microcytosis but does identify marked thrombocytosis.

K. Reticulocyte concentration (RC) (also called *absolute reticulocyte count* or just *reticulocyte count*)

1. The *RC* is the concentration of reticulocytes in blood expressed as number of reticulocytes per μL (or L) of blood.
2. RC equation (Eq. 3.3a):

$$RC = RP \times [RBC] \quad (\mathbf{3.3a})$$

$$CRP = RP \times \frac{\text{patient's Hct}}{\text{average Hct for species}} \quad (\mathbf{3.3b})$$

$$RPI = \frac{CRP}{RMT} \quad (\mathbf{3.3c})$$

3. Because the RC calculation requires a RP, it cannot be calculated when the RP is above an analyzer's analytical range (e.g., 24.5 % for the ADVIA 2120). Reanalyzing a diluted sample does not help because the RP remains the same. However, a minimum value can be calculated and is sufficient to indicate there is a marked reticulocytosis.

L. Reticulocyte percentage (RP) (sometimes called *reticulocyte count*, as is the RC)

1. The *RP* is the percentage of RBCs that are reticulocytes. For example, if there are 10 reticulocytes per 1000 RBCs, the RP is 1.0 %. The RP is used to calculate the RC and CRP (Eqs. 3.3a and 3.3b). If RP is not increased, an anemia is nonregenerative (or preregenerative); if RP is increased, an anemia may be regenerative or nonregenerative (see the following Section M, Corrected reticulocyte percentage).
2. Basics of procedures
   a. Microscopic (manual) procedure

(1) Equal volumes of blood and NMB stain are mixed and kept at room temperature for at least 15 min. NMB is a vital stain (i.e., it stains living cells) that penetrates RBCs and stains (and precipitates) cytoplasmic RNA. A smear of the blood-NMB mixture is made and air-dried for microscopic examination.

(2) The number of reticulocytes observed while counting 1000 or 2000 nonnucleated RBCs is recorded. For cats, some laboratories count only the aggregate reticulocytes, whereas other laboratories count aggregate and punctate reticulocytes and report each.

b. Automated techniques

(1) Flow cytometric methods

(a) In some analyzers (e.g., CELL-DYN 3700, ProCyte One), reticulocytes are enumerated by light scatter created from ribosomal RNA that has been stained and precipitated by NMB. The RP is determined by the percentage of RBCs with the light-scattering property. The CELL-DYN provides an immature reticulocyte fraction (IRF) based on those cells with enough RNA to be considered more immature.

(b) Some analyzers (e.g., Sysmex analyzers, ProCyte Dx) use a fluorescent dye, polymethine, to optically identify reticulocytes via their RNA content. Reticulocytes are classified into low, medium, and high-fluorescence populations. Sysmex RPs correlated well with a microscopic method for dogs; correlation was not as good for feline samples, running a little higher than the microscopic aggregate RP and typically much lower than the punctate RP when defining punctate reticulocytes as having 3–15 dots and no aggregates.[51] Unexplained false increases, sometimes marked, have been reported in stored canine samples.[22,52]

(c) ADVIA analyzers use a different stain, oxazine 750, to differentiate reticulocytes from mature RBCs (see Analytical Principles and Methods, Section III.E.2.d). They also have the ability to classify reticulocytes as most immature (high absorption), intermediate (medium absorption), or most mature (low absorption) reticulocytes based on their staining intensity; the most immature have the greatest staining and therefore greatest light absorption. The percentage and concentration of each fraction is determined.

(d) Flow cytometers identify fluorescent and nonfluorescent cells after staining reticulocytes with a fluorescent nucleic acid–binding dye (thiazole orange). The RP is the percentage of RBCs that are fluorescent.

(2) QBC-VetAutoread: Canine QBC RP is estimated by determining the thickness of RNA fluorescent cells that are at the top of the RBC layer and below the granulocyte layer.

(3) See Pseudoreticulocytosis (Anemia, Section II.A.3) for causes of false reticulocyte enumeration.

3. An increased RP does not always indicate a reticulocytosis, especially with moderate to severe anemias, because a relative increase may be due to a decrease in the concentration of mature RBCs rather than to an increase in the concentration of reticulocytes. Table 3.4 provides examples of RP data that illustrate this. Conversely, with mild or resolving anemias after an initial reticulocytosis, marrow may adequately respond without generating an increased RP; reticulocytosis is not seen but the Hct steadily increases. The circulating life spans of reticulocytes and the relationships between marrow stimulus and marrow responses (see Physiologic Processes, Section VI) need to be considered when blood reticulocyte data are interpreted.

Table 3.4. Evaluation of canine reticulocyte data

Canine	Unit	RI	Dog 1[a]	Dog 2[b]	Dog 3[c]
Hct	%	37–55	15	15	15
RP	%	0.0–1.0	2.0	9.0	0.0
CRP	%	0.0–1.0	0.7	3.0	0.0
[RBC]	$\times 10^6/\mu L$	5.5–8.5	2.0	2.0	2.0
RC	$\times 10^3/\mu L$	0–80	40	180	0

[a] Dog 1: Evidence of increased RBC production is not present. If the marrow is currently responding to the anemia, it is not reflected by the blood reticulocytes. The RP is increased because fewer RBCs, rather than more reticulocytes, are in the blood, i.e., RP is increased because the [reticulocyte] is WRI and the [RBC] is decreased.

[b] Dog 2: There is evidence of increased RBC production, and the marrow is currently responding to the anemia. The RP is increased because fewer RBCs and more reticulocytes are in the blood than there are in health, i.e., the RP is increased because the [reticulocyte] is increased and the [RBC] is decreased.

[c] Dog 3: There is no evidence of increased RBC production. Because reticulocytes were not seen, decreased RBC production may be one reason for the anemia; or, it could be too soon (< 3–4 d) for a reticulocyte response.

4. Microscopic determination of RP has relatively poor analytical precision, particularly when < 2000 RBCs are assessed.[22] Therefore, RP can be considered an estimate. Values that are calculated using the microscopic RP (i.e., RC, CRP, and RPI) can also be considered estimates.
5. The degree of increased polychromasia should correlate with the increase in feline aggregate RP and RP in most other species, particularly if polychromasia grading is based on fields of comparable RBC number rather than comparable thickness.

M. Corrected reticulocyte percentage (CRP) (also called *absolute reticulocyte percent* and *absolute % reticulocyte count*)
1. Calculation of CRP converts RP to a percentage that would estimate the RP if the animal were not anemic. This is necessary for appropriate interpretation of RP if RC is not calculated. If reticulocyte production was unchanged but anemia developed due to loss or destruction of mature RBCs, the RP would be increased because of a decrease in total RBCs, not because of increased reticulocyte production and release. The CRP determines whether the increased RP is enough to show regeneration or whether the increased RP may be due only to the anemia (Tables 3.4 and 3.5).
2. The CRP equation (Eq. 3.3b) corrects for anemia by comparing the patient's Hct to a mean Hct for the species and method. The CRP is needed only if the RP is increased, anemia is present, and the RC is not provided.
3. In theory, the RI for CRP should be the same as the RP RI. Therefore, if a CRP is greater than the RI for RP, there is a reticulocytosis.
4. Just as the RP must be corrected for the severity of the anemia, the degree of polychromasia needs to be interpreted with knowledge of the Hct, particularly if polychromasia is assessed in microscopic fields containing standardized RBC numbers rather than in fields of more comparable thickness and therefore volume (see Morphologic Features of RBCs, Sections I.A and B).

N. Reticulocyte production index (RPI) (also called *corrected reticulocyte count* and *reticulocyte index*)

Table 3.5. Evaluation of feline reticulocyte data

Feline	Unit	RI	Cat 1[a]	Cat 2[b]	Cat 3[c]	Cat 4[d]	Cat 5[e]
Hct	%	24–45	12	12	24	12	12
RP (aggregate)	%	0.0–1.0	6.0	2.0	0.6	1.2	0.1
RP (punctate)	%	0.0–10.0	30.0	30.0	30.0	6.0	1.0
CRP (aggregate)	%	0.0–1.0	2.0	0.7	0.4	0.4	< 0.1
CRP (punctate)	%	0.0–10.0	10.0	10.0	20.0	2.0	0.3
[RBC]	$\times 10^6/\mu L$	5.0–10.0	2.0	2.0	4.0	2.0	2.0
RC (aggregate)[f]	$\times 10^3/\mu L$	0–80	120	40	24	24	2
RC (punctate)[f]	$\times 10^3/\mu L$	5–500	600	600	1200	120	20

[a] Cat 1: There is evidence of increased RBC production. The aggregate reticulocytosis (↑ aggregate RC) indicates increased erythropoiesis. The punctate reticulocytosis (↑ punctate RC) is consistent with the aggregate response and may persist for 1–3 wk after the initial stimulus. In this case, the punctate CRP is a borderline percentage and thus difficult to interpret by itself.

[b] Cat 2: The duration of the anemia must be known to interpret the results. If the onset of anemia was within the past 2–3 d, then the results are consistent with the punctate reticulocytosis that occurs before an aggregate reticulocytosis. If the anemia has been present longer, the aggregate CRP and RC indicate a poor regenerative response.

[c] Cat 3: The magnitude of the punctate reticulocytosis indicates an Epo stimulus occurred at least 7–10 d ago and erythroid hyperplasia is present (resolving anemia). Absence of an aggregate reticulocytosis suggests that there is no longer an increased release of aggregate reticulocytes.

[d] Cat 4: Evidence of increased RBC production is not present despite an increased aggregate RP. Decreased production of RBCs is probably at least one reason for the anemia if there has been sufficient time for a marrow response.

[e] Cat 5: Evidence of increased RBC production is not present. Decreased production of RBCs is probably contributing to the anemia, as the RCs are WRIs.

[f] RIs for RC are based on data from the microscopic analysis of blood samples from 45 healthy cats (M.A.S. unpublished data).

1. During accelerated erythropoiesis, younger reticulocytes (called *shift reticulocytes*) may be released. In comparison to older reticulocytes, they may have mildly longer circulating life spans before becoming mature RBCs. Thus, the CRP may be increased because of the increased life span of reticulocytes, not because of increased production. Some people recommend the CRP be adjusted for the prolonged life spans by calculating the RPI, and some laboratories report a RPI.
2. Application of the RPI (Eq. 3.3c) requires that a RMT be known for each species in health and during accelerated erythropoiesis. RMT values frequently provided for human reticulocytes are as follows: When Hct = 45 %, RMT = 1.0 d; when Hct = 35 %, RMT = 1.5 d; when Hct = 25 %, RMT = 2.0 d; when Hct = 15 %, RMT = 2.5 d. For any Hct < 45 %, human RMT = (–0.05 × Hct) + 3.25. However, very few RMTs for domestic animals are known.
   a. In healthy dogs, reported values include a mean of 31 h, with an interval of 19–43 h. Values for anemic dogs are not known but probably are longer and thus RMTs in anemic dogs are probably different from RMTs in anemic people.
   b. In cats, reported RMTs (reticulocyte life spans) are about 12 h for Type II and III reticulocytes and 3.5 d for Type I (essentially punctate) reticulocytes,[21] and thus

use of the human RMT values is not appropriate for calculating a feline RPI.[53] Some have suggested that the prolonged punctate reticulocytosis occurring in cats after developing hemolytic or blood loss anemia may reflect a feline punctate RMT of as much as 10 d^9 rather than ongoing release of slightly immature RBCs throughout the time that increased erythropoiesis corrects the Hct back to baseline.

3. As rarely described in people with abnormal RBCs caused by a myelodysplastic syndrome, RMT may be prolonged (even > 20 d) because of impaired RNA degradation.[54] In such cases, the degree of reticulocytosis does not reflect the degree of regeneration or RBC immaturity.
4. If valid RMTs were available and used, an RPI > 1.0 typically would be evidence of a regenerative response. However, until the validity and clinical value of the RPI is established in a domestic species, the CRP or RC should be used to assess regenerative status. When interpreting the CRP and RP in an anemic animal, the possibility of a longer RMT should be considered. Evaluation of reticulocyte percentages and concentrations is presented in Tables 3.4 and 3.5.

MORPHOLOGIC FEATURES OF RBCs: CLINICAL SIGNIFICANCE AND PATHOGENESES

I. Assessment: Morphologic features of RBCs are evaluated as part of a routine CBC or as a separate procedure. For abnormalities in size, shape, and color, relative quantities of each abnormality per 100× objective microscope field are usually reported on a 3- or 4-plus scale. This microscopic assessment is subjective because of varied regions selected for evaluation, varied criteria for categorizing abnormalities, and varied grading systems. Two methods of selecting microscopic fields for assessment of traditional wedge smears are described here:
   A. By number of RBCs in field (provides an estimate relative to [RBC])
      1. If the features are always evaluated in regions of the blood film where RBCs occasionally touch one another but do not overlie one another, then the evaluation would be done in thicker areas of blood films from anemic animals as compared to nonanemic animals. With this approach, the number of RBCs per microscope field remains similar, but the volume of blood assessed per field is greater with anemia because more fluid volume was deposited in thicker regions of the smear. An increase in an abnormality per field of similar RBC density reflects an increased percentage of that abnormality, not necessarily an increased concentration. To use polychromasia as an example, a mildly increased percentage of polychromatophilic RBCs, as for a mildly increased RP, is not good evidence of a regenerative response without the anemia being considered as is done when calculating a CRP from a RP.
      2. With this approach, the assessment can be adjusted based on the number of RBCs per field. If a 1+ grade is based on finding six poikilocytes in a field of 400 RBCs, then a 1+ grade would be given if there were three poikilocytes in a field of 200 RBCs. This is still a relative system, so interpretation must include the severity of anemia (as for a CRP).
      3. Some grading systems have different grades based on the blood Hct. For example, the 1+ grade of poikilocytes (as just discussed) would apply if the Hct is 40 %, but six poikilocytes in a field of 150 RBCs would be a 1+ grade if the Hct is 20 % (similar to the CRP system).
   B. By thickness of blood film area (may better estimate concentration in blood)

1. If features are always evaluated in the optimal zones of wedge blood films, typically about 1–2 10×-field diameters behind the feathered edge where WBCs are well spread (like fried eggs), then fewer RBCs per field will be present in blood from anemic animals, but the deposited volume of blood will be similar to that in the optimal zone of blood from a nonanemic animal. With this approach, an increase in an abnormality more closely reflects an increased concentration (rather than %) of that abnormality, so, for example, a mild increase in polychromasia is supportive of a regenerative response (more reticulocytes per blood volume).
2. This method requires appropriate thinning of smears and recognition of areas of similar smear thickness (optimal zones). This is typically based on the subjective appearance of the cells which "ball up" in thicker areas, lie flat in the optimal zones, and are broken in areas that are too thin (feathered edge). The precise location of the optimal zone of a blood film varies with the sample and technique (e.g., protein concentration and length of blood film).

II. General features
   A. *Discocytes*: Mature RBCs of noncamelid domestic mammalian species are disks with different degrees of biconcavity that creates central pallor where the cells are thin enough (Table 3.2).
   B. *Rouleau, rouleaux* (pl.) (French for "roll" and "rolls"): a linear branching or nonbranching aggregate of RBCs resembling a roll of coins (Figure 3.7A)
      1. Rouleaux are common in some species (especially horses and cats).
      2. Formation of rouleaux involves charge interactions between RBC membranes and plasma macromolecules and is therefore affected by RBC factors (shape and membrane composition), albumin factors (glycation), globulin factors (charge, size, and number), lipid content of plasma and perhaps RBC membranes, pH (affects cell and protein charges), and exogenous macromolecules (dextrans).[55]
      3. Rouleaux tend to increase if there is hyperglobulinemia or hyperfibrinogenemia. An increase is most evident when rouleaux extend to near the feathered edge of a blood film and thus reduce the width of the optimal zone (appropriate part of the monolayer area).
      4. Rouleaux may occur in vivo and contribute to blood hyperviscosity, thus decreasing effective blood flow and tissue oxygenation.

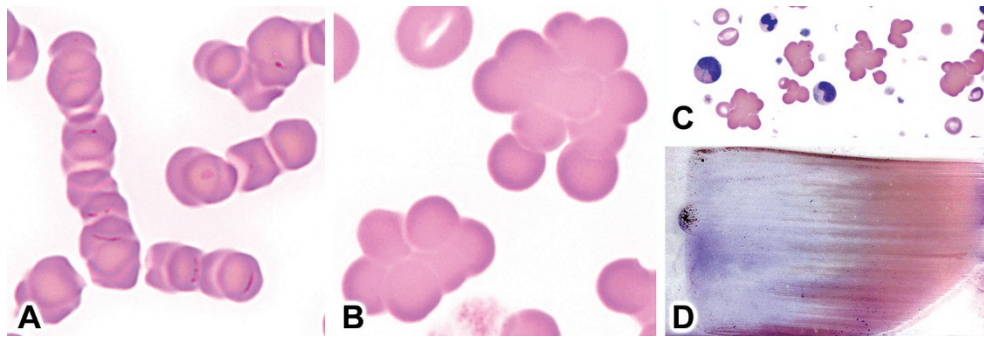


Figure 3.7. A. Rouleaux, horse. B–D. Agglutination, dog. RBC agglutination may cause clearing in thick areas of a blood smear as seen at lower magnification (C) and grossly with the appearance of a so-called "reverse" smear (D).

C. *Agglutination* (*erythroagglutination*): the clumping of RBCs to form grapelike clusters held together by an agglutinin, commonly an immunoglobulin; often called *autoagglutination* when occurring spontaneously (Figure 3.7)
   1. Autoagglutination is seen in many immune-mediated hemolytic anemias, in cold agglutinin disorders (rare), with anticoagulant-induced antibody binding, and occasionally in animals without evidence of hemolysis.[56] The agglutinin typically is a cold antibody, i.e., an antibody that has maximal activity below body temperature (often 4–20 °C). It may be accompanied by warm antibodies that contribute to anemia in vivo. IgM and/or IgG can mediate agglutination.[57]
   2. Autoagglutination is seen best in thicker portions of a blood smear rather than where most blood smear evaluation is done. Marked agglutination may make these regions appear grossly thinner (paler) while nonagglutinated cells form a thicker region than usual near the feathered edge, a so-called reverse smear (Figure 3.7D).
   3. The RBC clusters formed by autoagglutination must be differentiated from rouleaux, which classically appear microscopically as stacks of RBCs but can appear as grape-like clusters and mimic agglutinates when rouleaux collapse on themselves. Grape-like clusters in the presence of prominent rouleaux are not good evidence of agglutination. When examined macroscopically in a tube or as a drop on a slide, blood containing agglutinated RBCs or substantial rouleaux may have a fine to coarse grainy appearance.
   4. *Saline dispersion test*: One method used to differentiate antibody-mediated agglutination from rouleaux or other causes of agglutination is the saline dispersion (dilution) test, in which blood is diluted with saline and a wet preparation of the diluted blood is examined to see if RBC clumps disperse. Dispersion, assessed macroscopically or microscopically, supports rouleaux rather than autoagglutination. Although commonly called a "saline agglutination test," this test was meant to assess dispersion of RBC clumps that are already apparent, not to look for agglutination when clumping is not apparent. A positive result is evidence of agglutination and IMHA, but it is not diagnostic of IMHA.
      a. Rouleaux should disperse into individual RBCs after dilution of blood with enough saline. A dilution of one part blood to one part saline (1:2 dilution) may disperse rouleaux, but much greater dilutions may be needed to disperse rouleaux (Figure 3.8). At least one part blood to three parts saline (1:4 dilution) is preferable to a 1:2 dilution starting point, but complete saline replacement of plasma

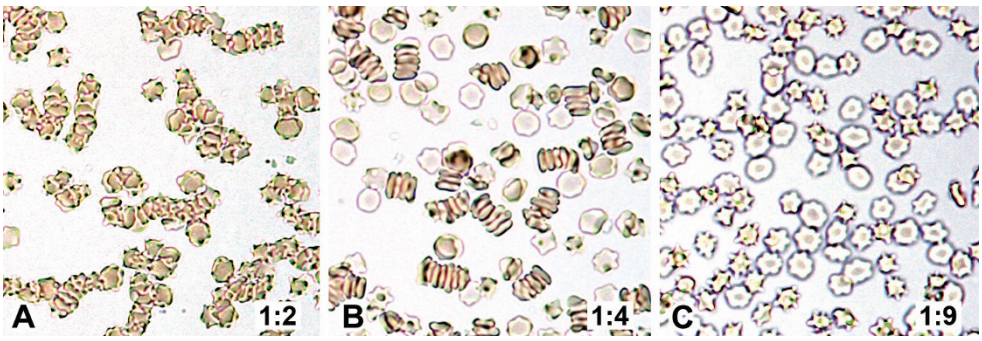


Figure 3.8. Saline dispersion of rouleaux in nonstained wet preparations, dog. A. Marked rouleaux, blood diluted 1:2 in saline. B. Rouleaux, same blood diluted 1:4 in saline. C. Absence of rouleaux, same blood diluted 1:9 in saline.

is needed to eliminate plasma factors that promote RBC interactions. In one study, increasing the dilution to 1:49 decreased positive test results; with this dilution, 4 of 138 dogs judged not to have IMHA were still considered to have microscopic agglutination, but the cause of clumping was not investigated (cold-reacting antibody?, strong rouleaux?) and it is not known if it would have disappeared with washing.[58] In many cases, RBC washing eliminates what appears to be agglutination, and DAT results are negative.[59,60] Although autoantibodies are not expected to wash off, weak-binding antibodies or antibodies binding to removable RBC-adherent molecules have not been completely excluded.

   b. This test is often done in the clinical setting by clinicians or clinical staff with limited training in hematology and microscopy, but it is also done by trained laboratory technicians. In either setting, it lacks standardization, e.g., volumes of blood and saline, type of saline, sample and incubation temperature, timing of evaluation, method of evaluation, definition of agglutinates, quality controls. False positive interpretations, i.e., agglutination concluded when not present, can misdirect patient management.

5. Heparin can induce agglutination of equine RBCs.
6. In vitro EDTA-induced agglutination has been reported in a cat.[61] Agglutination appeared to be mediated by IgM, which likely bound to epitopes exposed in the presence of EDTA.
7. Hematology analyzers may generate RBC scatterplots characteristic of agglutination when present in some samples. With the ADVIA series, small agglutinates appear as a macrocytic normochromic cloud straight above and distinct from the normocytic normochromic cloud (Figure 3.6D). Macrocytic cells from other causes form more of a continuum from normocytic to macrocytic normochromic or macrocytic hypochromic regions.
8. Agglutination may interfere with the electronic or optical evaluation of RBCs when small groups of RBCs pass through the counting chamber as "large cells." In such cases, the measured MCV and [RBC] are erroneous (unless programs exclude outlier values), as are the values calculated from those measured values. Automated RCs also may be unreliable.

D. Rubricytosis (increased nRBCs, metarubricytosis, normoblastemia) (Figure 3.9)

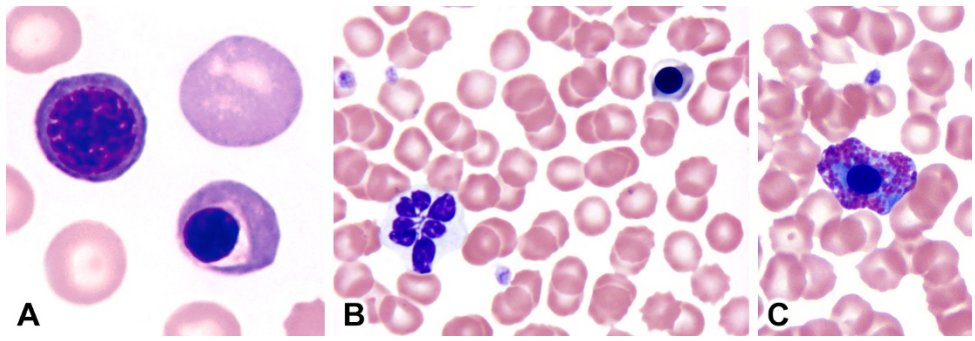


Figure 3.9. A. Appropriate rubricytosis with a rubricyte, a metarubricyte, a polychromatophilic macrocyte, and a normocytic discocyte, dog. B and C. Inappropriate rubricytosis with the heatstroke triad of a hypersegmented neutrophil, metarubricyte, and eosinophil with pyknotic nucleus, dog with seizures.

1. *Rubricytosis* is an increased [nRBC] in blood. The term is not specific for cells of the rubricyte stage, referring instead to all stages of erythroid precursors, as they all include "rubri" when using the rubricyte nomenclature system. Usually, most are metarubricytes, fewer are rubricytes, and younger precursors are rarely seen. Cells in stages prior to metarubricytes may be in mitosis. All stages are normoblasts using the normoblast nomenclature system.
2. An increase in nRBC/100WBC may not be an increased [nRBC] if leukopenia is present. Most healthy adult dogs have 0 nRBC/100WBC, but few have 1 nRBC/100WBC, and greater numbers of nRBCs are expected during the first month of life. Llamas and alpacas may have up to 3 nRBC/100WBC in health.
3. Rubricytosis is common in regenerative anemias but also occurs in nonregenerative anemias and in nonanemic animals without reticulocytosis. Therefore, rubricytosis should not be considered a consistently reliable indicator of a responsive marrow.
   a. *Appropriate rubricytosis* is rubricytosis concurrent with regenerative anemia (with reticulocytosis) in dogs, cats, cattle, pigs, alpacas, llamas, and occasionally in horses. As for reticulocytes, nRBCs are released in response to an increased Epo stimulus during accelerated erythropoiesis.
   b. *Inappropriate rubricytosis* is rubricytosis in the absence of reticulocytosis and an Epo stimulus; it may be present with a nonregenerative anemia or in the absence of anemia. It occurs primarily when there is loss of controlled release of nRBCs from marrow or other erythropoiesis sites, e.g., the spleen: nRBCs egress without nuclear extrusion and before maturing to reticulocytes. Inappropriate rubricytosis may be present in a variety of disorders.
      (1) Marrow damage: nRBCs may gain entrance into marrow sinusoids through endothelial damage caused by necrosis, inflammation, endotoxemia, nonhemic or hemic neoplasms (particularly myelodysplastic syndrome with erythroid predominance in cats), chemotherapy (e.g., vincristine),[62] heatstroke,[63] or hypoxia.
         (a) Atypical or early-stage nRBCs suggest a hemic neoplasm or drug-induced rubricytosis (e.g., vincristine).
         (b) The triad of (i) inappropriate rubricytosis, (ii) dead WBCs with pyknotic nuclei, and (iii) botryoid hypersegmentation (neutrophils, eosinophils) occurs in dogs or cats with heat stroke, e.g., trapped in hot car or clothes dryer, seizures, Adderall toxicity (Figure 3.9).
      (2) Extramedullary hematopoiesis (especially splenic): This may allow release of cells before nuclear extrusion.
      (3) Splenic contraction: Splenic blood contains nRBCs that are completing maturation, in addition to those forming there.
      (4) Splenectomy: The few nRBCs that are normally released from marrow are not "caught" by the spleen.
      (5) Lead poisoning in dogs: The cause is not clear.
      (6) Certain inherited dyserythropoietic disorders including bone marrow dyscrasia in poodles with macrocytosis (see Other Nonneoplastic Leukocyte Disorders, Section II, in Chapter 2),[64,65] dyserythropoietic anemia and myopathy syndrome in English springer spaniels or Labrador retrievers,[66,67] congenital dyserythropoiesis of polled Hereford calves,[68] and congenital dyserythropoietic anemia in Murray Grey calves[69]
      (7) Severe cobalamin deficiency

c. Multiple concurrent mechanisms may be involved. For example, with cancer treatment, there may be direct effects of the drug on erythroid cells or marrow microenvironment, damage secondary to proliferating or dying neoplastic cells in the marrow, impaired splenic function from neoplastic involvement of the spleen, responsive (appropriate) erythropoiesis, or other impacts of complications related to the neoplastic state.

III. RBC color

A. *Central pallor* refers to the pale central region of a RBC that is due to the relative thinness of the area created by the cell's biconcave shape. In health, the degree of central pallor varies with the species (Table 3.2), the individual, and the spreading of the cells in a blood film.

1. Increased central pallor, when not artifactual (torocytes), is indicative of hypochromasia. This visible hypochromasia is most apparent in dogs and camelids with Fe-deficiency anemia, in which case the cells contain decreased Hgb mass per cell, not just a decreased Hgb concentration per cell. In camelids, the pallor of hypochromasia is often unevenly distributed (e.g., eccentric or bipolar).
2. Decreased central pallor usually indicates abnormally shaped RBCs (poikilocytes, including spherocytes). It is also present near a blood film's feathered edge because of artifactual distortion of RBC shape.

B. A *ghost cell* is an extremely pale-staining RBC consisting primarily of cell membrane with little or no residual cytoplasmic Hgb (Figure 3.10A).

1. Ghost cells are often formed by complement-mediated intravascular hemolysis; membrane attack complexes form membrane pores through which Hgb molecules leak out. Ghost cells also arise from other causes of membrane damage, including certain envenomations, and from osmotic swelling. In oxidative hemolytic anemias, ghost cells may contain Heinz bodies (the denatured Hgb cannot escape).
2. Ghost cells may form in vitro as a result of smearing trauma. These artifactual ghost cells are often distorted or ruptured, appearing beside a smudge of Hgb. Ghost RBCs may form from osmotic lysis within a hematology analyzer, in which case they may be evident on a scatterplot but not seen on a blood smear.

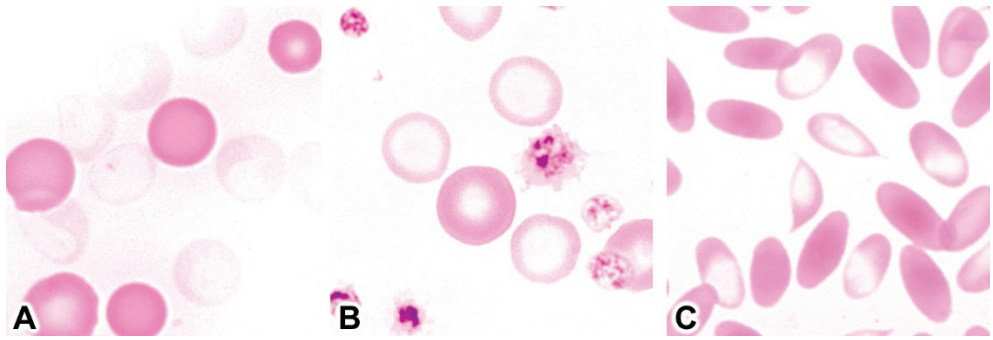


Figure 3.10. A. Ghost RBCs with little residual Hgb and hyperchromic spherocytes without central pallor, dog with IMHA. B. Hypochromic microcytic RBCs, platelets, and a typical discocyte, dog with Fe deficiency. C. Hypochromic RBCs including a dacryocyte and a spindle cell, Fe-deficient alpaca.

C. A visually *hypochromic RBC* is a poikilocyte (leptocyte and/or codocyte) with increased central pallor and, in camelids, an uneven distribution of Hgb forming nonstaining regions centrally or at one or both poles of the cell (Figure 3.10B and C). Central pallor is not abrupt as in hypochromasia-mimicking torocytes, and the Hgb that does stain tends to stain paler than that in torocytes or RBCs of healthy animals.
   1. *Hypochromasia* is an increased number of hypochromic RBCs, which may be reflected by decreased MCHC and/or CHCM if the hypochromic population is large enough.
   2. Hypochromic RBCs result from a decreased intracellular [Hgb]. When visually prominent (microscopically), they usually are associated with microcytic Fe-deficiency anemia and decreased MCH (less Hgb to stain); milder visual hypochromasia may occur with portosystemic shunts. In contrast, hypochromasia based on the MCHC or CHCM alone (without microscopically apparent hypochromic RBCs) is usually associated with reticulocytosis (i.e., regenerative anemias), reticulocytes tending to have increased Hgb to stain (MCH is WRI or increased) but decreased Hgb concentrations (MCHC and CHCM) because of disproportionately greater volumes that decrease the concentration of Hgb per cell.
   3. Hypochromic RBCs of Fe deficiency are prone to structural changes including loss of discocyte shape (ovalocytes, codocytes) and fragmentation with formation of keratocytes and schistocytes.

D. A *polychromatophilic RBC (polychromatophil)* is an anucleate, immature erythroid cell (reticulocyte) with enough cytoplasmic RNA to stain bluish to purplish with a Wright stain (Figures 3.9A and 3.11D). Polychromasia can refer to the appearance of an individual RBC (i.e., a cell with polychromasia) or a population of RBCs (i.e., increased polychromasia). In camelids, polychromatophils may be less elliptical than mature RBCs and even round like their precursors.
   1. Polychromatophilic RBCs would appear as aggregate reticulocytes after vital staining with NMB. A cell's polychromasia (multiple colors) is the result of its cytoplasmic RNA (basophilic staining) and Hgb content (eosinophilic staining).
   2. If the concentration of polychromatophilic RBCs in blood is increased, there is increased polychromasia that reflects accelerated erythropoiesis. However, routine subjective assessment of polychromatophilic RBCs in blood films may better reflect the percentage of polychromatophilic RBCs, in which case, depending on how it is done (see Morphologic Features of RBCs, Section I), increases should be interpreted as for RP, in light of the degree of anemia.

E. A *reticulocyte* is an anucleate, immature erythroid cell with stainable cytoplasmic RNA (Figure 3.11).
   1. A reticulocyte's cytoplasmic RNA may be visualized after staining with NMB stain or other vital stains (brilliant cresyl blue, crystal violet). In cats, reticulocytes are grouped into two types (aggregate and punctate) based on the staining pattern (amount) of the RNA. Punctate reticulocytes are generally present in insignificant numbers in other species.
   2. A reticulocyte's cytoplasmic RNA may also be visualized after staining with Wright stains, in which case reticulocytes appear as polychromatophilic RBCs, which is what they are typically called in that context. All polychromatophilic RBCs are reticulocytes, but not all reticulocytes have enough RNA to appear as polychromatophilic RBCs.
   3. Reticulocytosis (increased blood RC) is an important indicator of accelerated erythropoiesis, and it is more objective than assessment of polychromasia.

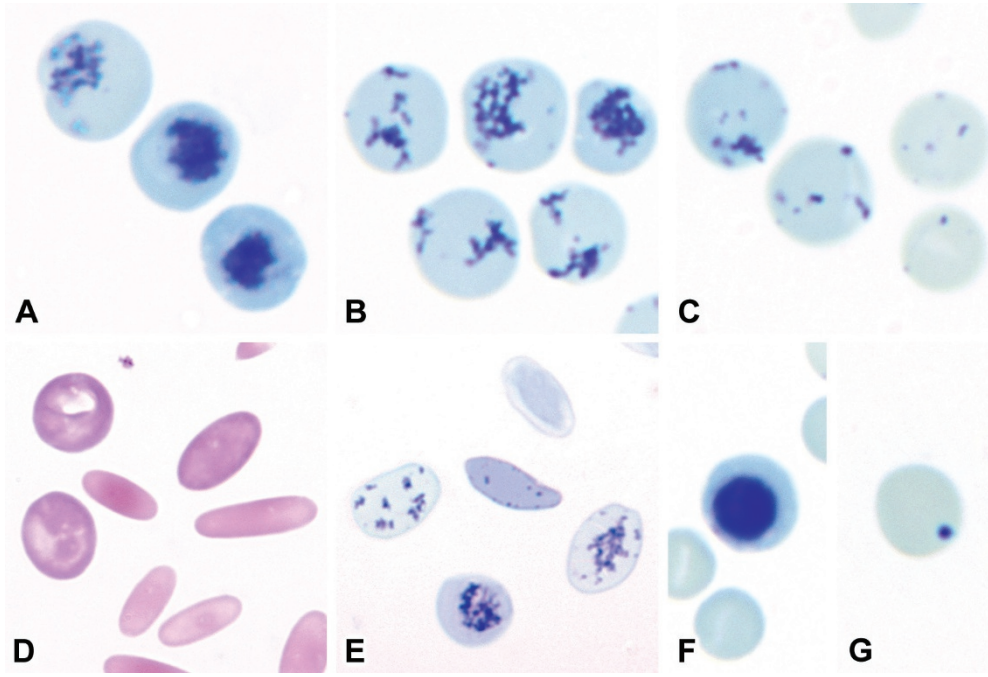


Figure 3.11. A–C. Continuum from immature aggregate reticulocytes to punctate reticulocytes, cat, NMB vital stain. At least three of the four cells in C contain too little RNA to appear polychromatophilic with a Wright stain. D and E. Alpaca blood with three round to elliptical polychromatophilic RBCs (D, Wright stain) and similar cells after NMB vital staining (E) showing progression of aggregate to punctate reticulocytes. Vital NMB staining of a feline nRBC (F) and Howell–Jolly body (G).

IV. RBC organisms
   A. Identifying features are listed in Table 3.6 and shown in Figure 3.12.
   B. Major aspects of the anemias or disorders caused by organisms are included in the Nonregenerative Anemia and the Hemolytic Anemias sections.

V. Inclusions other than organisms (Table 3.7 and Figure 3.13)
   A. Basophilic stippling (punctate basophilia) (Figure 3.13A)
      1. *Basophilic stippling* is the presence of fine to coarse, basophilic dots of aggregated ribosomes (RNA) dispersed relatively homogeneously throughout the RBC cytoplasm. Basophilic stippling must be differentiated from siderotic granules, which are usually located in clusters.
      2. Basophilic stippling is seen with regenerative anemias, especially in ruminants, but also in some dogs and cats. When seen without corresponding polychromasia or reticulocytosis, or in nonanemic animals, plumbism is a differential, especially in dogs. Lead inhibits the pyrimidine 5′-nucleotidase that helps degrade nucleotides in RNA, and this enzyme is less active in some species (e.g., ruminants) and individuals.
   B. Cabot rings (Figure 3.13B)
      1. Cabot rings are filamentous peripheral rings, likely consisting of microtubules and associated histone proteins,[70,71] that can be found in immature erythrocytes

Table 3.6. RBC organisms: identifying features and associated pathogenic processes (Figure 3.12)

Organism	Identifying features[a]	Associated pathogenic processes
Anaplasma marginale (cattle)	Marginal body is a dark-staining coccus about 0.5 μm in diameter on the internal margin of RBCs; typically one organism per cell but may be multiple	Immune hemolysis, possibly others
A. centrale (cattle)	As for *A. marginale*, but located centrally	Immune hemolysis, possibly others
Babesia spp. (all species)	Intracellular oval to teardrop or pear-shaped (pyriform) trophozoites (piroplasms); sizes vary with species (see the text); typically nonstained to pale blue with a darker outer membrane and a reddish or purple eccentric nucleus	Theories: immune mechanisms, protease activity, decreased cell pliability, oxidative damage
Cytauxzoon felis[b] (cats)	Intracellular oval structures (0.1–2.0 μm) with outer thin rim and eccentric nucleus; may resemble signet ring or safety pin; one to several piroplasms per cell	May be multifaceted (anemia of inflammation, marrow damage; possibly hemolysis)
Mycoplasma[c] spp. of cattle	Basophilic rings, rods, or cocci (0.3–1.0 μm) on surface (top or edge) of RBCs	Immune hemolysis
Mycoplasma haemocanis[d,e] (dogs)	Typically thin chains of basophilic cocci on membrane that may form pleomorphic patterns (violin bow, figure-8, oval, cross); occasionally seen as individual cocci or rods	Immune hemolysis
Mycoplasma haemofelis[f] (cats)	Typically basophilic cocci (individual or in short chains) and rings (< 1 μm) on RBC surface (top or edge)	Immune hemolysis
"*Candidatus* Mycoplasma haemominutum"[f] (cats)	Individual or short chains of basophilic cocci (0.1–0.3 μm) present on RBCs (top or edge)	Immune hemolysis (but has low virulence)

Continues

Table 3.6. RBC organisms: identifying features and associated pathogenic processes (Figure 3.12) (Continued)

Organism	Identifying features[a]	Associated pathogenic processes
"*Candidatus* Mycoplasma haemolamae"[g] (alpacas, llamas)	Few to numerous basophilic cocci and rings (~0.5 μm) on RBCs (top or edge) and between RBCs	Immune hemolysis
Rangelia vitalii (dogs)	Few round to ovoid (~2 × 3 μm) or teardrop piroplasms with eccentric purplish nuclei and pale cytoplasm; 1–2/RBC and one to several in neutrophils or monocytes	Immune hemolysis
Theileria spp. (cattle, horses)	Highly pleomorphic purplish piroplasms including cocci, rings, rods, pears, and Maltese crosses	Several theories, as for *Babesia* above

[a] Appearance as seen on a Wright-stained blood film unless stated otherwise
[b] A genetically distinct *Cytauxzoon* species may infect cats in Europe and Asia.
[c] Basonym, *Eperythrozoon*; *M. wenyonii* and *Candidatus* M. haematobovis are reported
[d] Basonym, *Haemobartonella canis*
[e] "*Candidatus* Mycoplasma haematoparvum" also occurs in dogs (see the text); described as singlet or rarely doublet 0.3 μm cocci
[f] Basonym, *Haemobartonella felis*; "*Candidatus* Mycoplasma turicensis" infects cats but is not expected to be seen in blood smears, and *Candidatus* Mycoplasma haematoparvum or similar organism has been reported in cats based on molecular testing
[g] Previously considered an *Eperythrozoon* sp. of alpacas and llamas

(reticulocytes and nRBCs) and less often in mature erythrocytes. They are reddish with May–Grünwald Giemsa staining and blue with vital new methylene blue staining. Some fold into figure-8 shapes.

2. They have been reported in a dog with dyserythropoiesis and multilineage dysplasia,[72] and occur in people with various primary and secondary myelodysplastic disorders, lead poisoning, or severe infections. In camelids, similar rings (presumably Cabot rings) have been called marginal bands and consist predominantly of a circumferential band of about 10–20 (as many as 55) microtubules. In health, they are reportedly present in about 3 % of blood RBCs, mostly reticulocytes, and they also appear in late-stage marrow erythroid precursors.[71,73] In disease, they may be numerous. Many Cabot rings were present in an alpaca with a moderately regenerative hypochromic anemia supportive of Fe deficiency; they were in 55 % of RBCs, almost all the reticulocytes, and many of the more-mature RBCs.[74] They were seen with vital new methylene blue staining but not with Wright staining.
3. Circumferential marginal bands are present in fetal RBCs of at least some mammals, and they are present in the elliptical nucleated RBCs of fish, birds, reptiles, and amphibians (and in the anucleate RBCs of amphibians that lack nRBCs).[73] In amphibians, they have

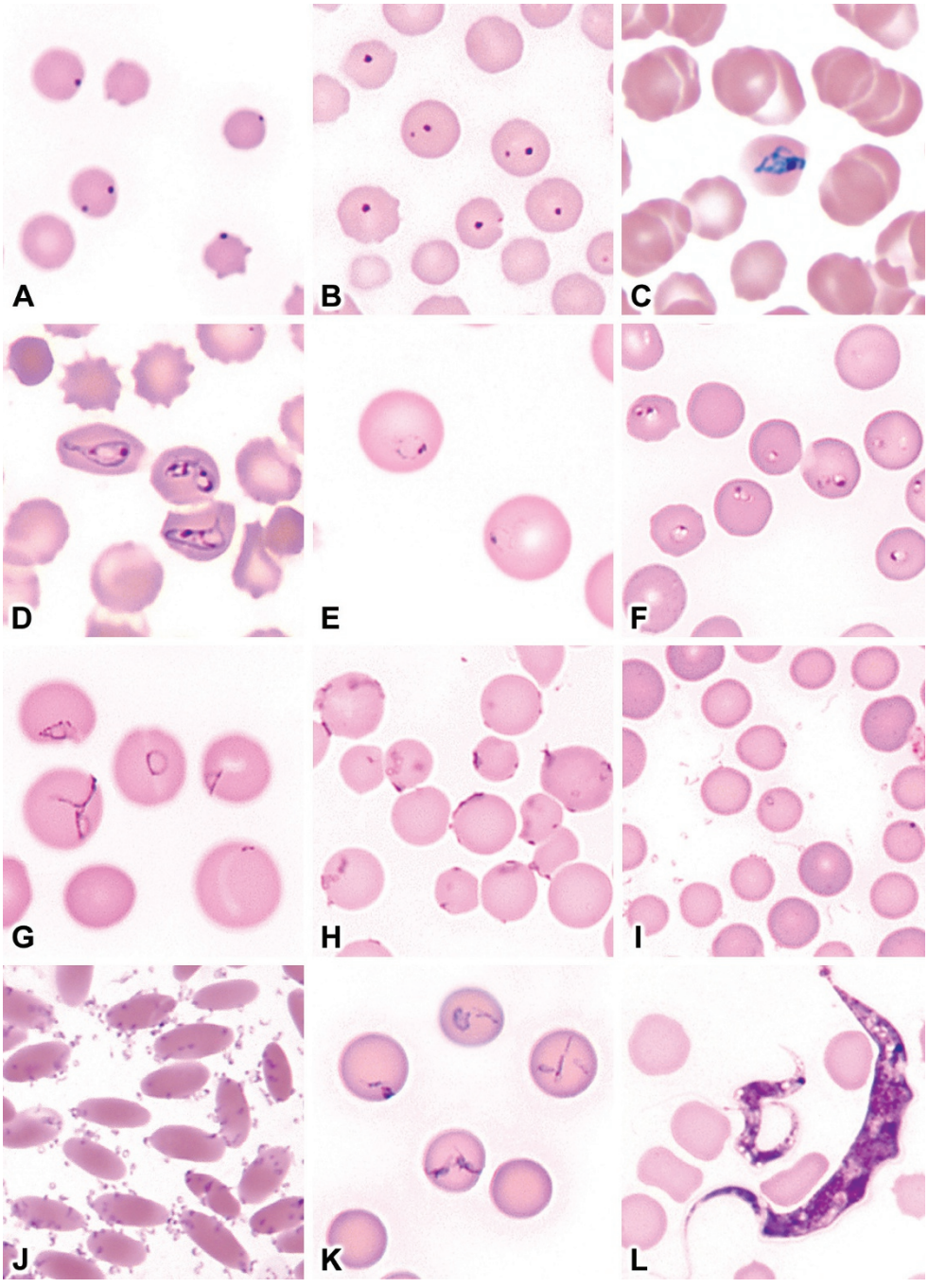


Figure 3.12. A. *Anaplasma marginale*, cow. B. *Anaplasma centrale*, cow. C and D. *Babesia canis* variations, dogs. E. *Babesia gibsoni*, dog (from ASVCP slide contributed by Dr. A.R. Irizarry-Rovira, et al., 1999). F. *Cytauxzoon felis*, cat. G. *Mycoplasma haemocanis*, dog. H. *Mycoplasma haemofelis*, cat. I. *Mycoplasma wenyonii*, most detached from RBCs, cow (from ASVCP slide contributed by Dr. E.G. Welles, et al., 1993). J. *Candidatus* Mycoplasma haemolamae, alpaca cria. K. *Theileria buffeli*, cow. L. *Trypanosoma theileri*, cow (from ASVCP slide contributed by Dr. H. Bender, et al., 1989).

Table 3.7. RBC inclusions other than organisms: features, clinical significance, and associated pathogeneses (Figure 3.13)

Inclusions	Identifying features[a]	Clinical significance	Associated pathogeneses
Basophilic stippling*	Fine to coarse, blue to dark purple dots of relatively dispersed aggregated ribosomes	Regenerative anemia (especially cattle, some dogs and cats); plumbism	Young cells; plumbism (inhibition of pyrimidine 5′-nucleotidase)
Cabot ring	Thin peripheral rings or folded figure-8 loops, mostly in immature RBCs; reddish with May–Grünwald Giemsa stain, blue with NMB stain	Suggests dyserythropoiesis; seen in healthy camelids in low numbers	Unclear; likely persistence of microtubules and associated histone proteins
Distemper in dogs	< 0.3–3 μm, homogeneous, irregular or round, magenta (Diff-Quik) or pale blue (Wright) bodies; also in WBCs	Indicates active distemper infection; rare finding wherever distemper is controlled by vaccination	Inclusions consist of nucleocapsids, not complete viruses
Heinz body*	Rounded, mildly pale Hgb-colored, structures that protrude upward or peripherally and may occur as free bodies; baby blue with NMB stain	Exposure to endogenous or exogenous oxidants; defective RBC reducing pathways (rare)	Oxidants overwhelm reductive capacity of RBC; oxidized Hgb precipitates and binds to RBC membrane
Hemoglobin crystal	Deeply stained condensed Hgb that forms elongate rectangular, rod, or other polyhedral shapes within RBCs	None known in domestic mammals; common in camelids; may develop in stored (refrigerated) blood samples	Occurs with hemoglobinopathies in people and perhaps other species
Howell–Jolly body*	Usually a homogeneous, dark purple–staining, round body midway from the RBC center to the edge; can be ring-like (especially in cats)	Increased erythropoiesis, decreased splenic function, certain drugs that affect mitosis (e.g., vincristine)	Nuclear remnant that remained free in the cytoplasm after mitosis; persists in RBC if the spleen does not pit it
Siderotic granules*	Loose aggregate of fine to coarse granular basophilic inclusions; stain blue with Prussian blue stain for Fe	Excess Fe in body; plumbism in dogs; myeloproliferative neoplasms, regenerative anemias, idiopathic	Fe accumulates in damaged mitochondria or in autophagocytic vacuoles

* A relatively common inclusion

Note: Basophilic stippling is more common in cattle than in dogs and cats, and it is not expected in horses.

[a] Appearance as seen on a Wright-stained blood film unless stated otherwise

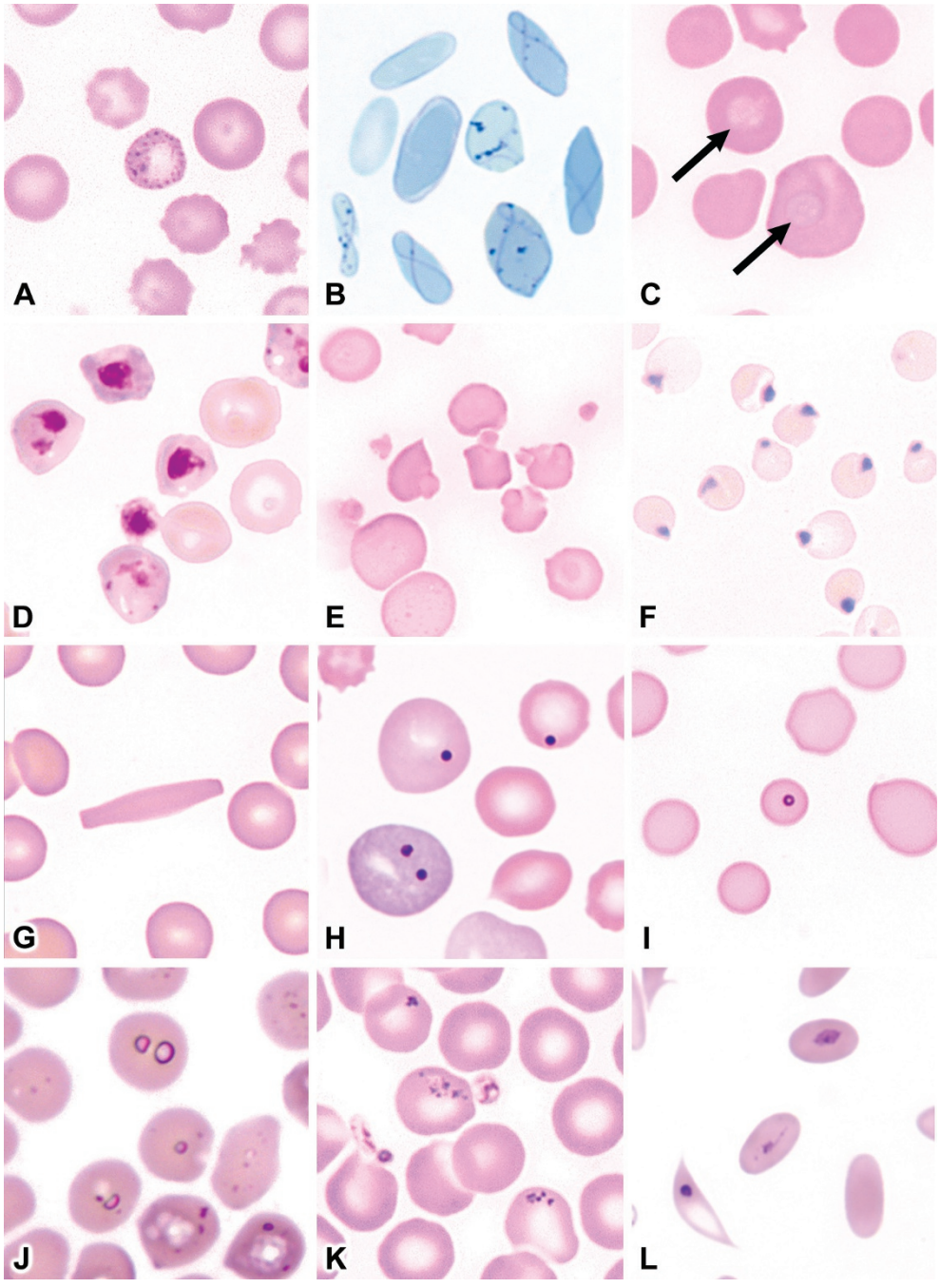


Figure 3.13. RBC inclusions. A. Basophilic stippling of plumbism, dog. B. Cabot rings, alpaca, vital NMB stain. C. Distemper inclusions, dog (from ASVCP slide contributed by Dr. D.C. Bernreuter, 1981). D. Distemper inclusions, Diff-Quik stain, dog (from ASVCP slide contributed by Dr. J.R. Duncan, 1981). E. Heinz bodies, cat. F. Heinz bodies, vital NMB stain, cat. G. Hemoglobin crystal, dog. H. Single and dual Howell–Jolly bodies in mature and polychromatophilic RBCs, dog. I. Howell–Jolly body, ring variant, horse. J. Refractile artifact, Diff-Quik stain with water in fixative, dog. K. Siderocytes with siderotic granules, dog. L. Unidentified basophilic inclusions, alpaca.

been hypothesized to form from the fusion of two peripheral and opposing arcs of microtubule bundles that are responsible for the progression of developing erythroid cells from round precursors to doubly pointed ("spindle cells") to singly pointed (spatulate cells or dacryocytes) to elliptical cells as the bundles fuse first at one end and then at the other.[75]

C. Distemper inclusions in dogs (Figure 3.13C and D)
   1. RBC distemper inclusions are rare findings where distemper is well controlled by vaccination. These aggregates of nucleocapsids may occur in the early viremic stage and before clinical illness in dogs. They may appear as pink or pale blue amorphous inclusions of varying shapes and sizes. They are more easily detected with quick-dip stains (e.g., Diff-Quik) than with Wright stains.
   2. Similar cytoplasmic inclusions can be present in blood neutrophils and lymphocytes (see Chapter 2).

D. Heinz bodies (Figure 3.13E and F)
   1. *Heinz bodies* are aggregates of denatured Hgb caused by oxidative damage. In Wright-stained blood films, Heinz bodies that are large enough to see have nearly the same staining features as normal Hgb but appear as slightly pale structures that may protrude from the edges of RBCs. Some may be detached.
   2. Heinz bodies are visualized with NMB stain as pale blue (baby blue), rounded structures associated with RBC membranes. They can be single or multiple, and their size varies from pinpoint to larger and protruding.
   3. Except in cats, the presence of Heinz bodies in an animal with a hemolytic anemia indicates Heinz body–induced hemolysis. Few to > 50 % of RBCs may contain single Heinz bodies in cats without anemia or clinical evidence of disease.[76]

E. Hgb crystals (Figure 3.13H)
   1. These are commonly encountered in blood films of alpaca and llama blood, and they are seen occasionally in blood films from other domestic mammals including dogs and cats. They have no known diagnostic significance. Rarely, they have been seen transiently in young anemic cats. Hgb electrophoresis has failed to demonstrate abnormal Hgb molecules in domestic mammals that have had Hgb crystals. Some may form in vitro because of sample storage conditions and aging.
   2. Commonly, Hgb crystals are long, narrow rectangles that stretch and distort the RBCs. In camelids, they typically appear as dark, contracted RBCs because of their more compact polygonal shapes as seen clearly by electron microscopy. Formation in camelids may relate to the greater MCHC of camelid RBCs and therefore perhaps a propensity to precipitate.

F. Howell–Jolly bodies (Figure 3.13H and I)
   1. A *Howell–Jolly body* is a nuclear remnant that has remained free in the cytoplasm after mitosis of a RBC precursor. The Howell–Jolly body is a whole chromosome or chromosome portion that was not incorporated into a new nucleus during mitosis. Although only reported when increased in blood RBCs, they are also seen in some nRBCs. Howell–Jolly bodies are referred to as micronuclei in the context of toxicologic pathology and the micronucleus test to assess potential genotoxic compounds.
   2. Howell–Jolly bodies can be found in healthy mammals, frequently in cats and occasionally in dogs and horses. There is usually one per RBC. The number of blood RBCs containing Howell–Jolly bodies increases during accelerated erythropoiesis and

also may increase in mammals with decreased splenic function (including after splenectomy). Increased typical, atypical, single, or multiple Howell–Jolly bodies may be present in dyserythropoietic conditions, including after administration of vincristine, which impairs mitotic spindle function.[65,77]

G. Refractile artifacts (Figure 3.13J)
   1. RBC refractile artifacts are frequently found in stained blood films. Objects are *refractile* when they change from dark to shiny as the focal plane is changed; refractile artifacts are recognized by focusing up and down and assessing for this property. They may appear as crescents or as small to large, irregular shapes within the RBCs. The defect that creates the refractile structure develops during the drying or staining of the RBCs. Refractile artifacts can be numerous when H_2O contaminates a dip-stain fixative, which is largely methanol, a hygroscopic chemical that attracts H_2O from ambient air.
   2. When refractile artifacts are in a plane of focus that makes them resemble black structures, they can be confused with RBC inclusions (e.g., parasites).
   3. Refractile artifacts are different from erythrocyte refractile (ER) bodies as described by Schalm.[78] RBC refractile bodies are Heinz bodies seen on air-dried blood films by using a wet NMB stain under a cover glass. In these preparations, Heinz bodies appear as refractile bodies in RBCs; the bodies are dark foci in one focal plane but become shiny in another. Heinz bodies are not refractile in films stained with a Romanowsky-type stain (e.g., Wright, Wright–Giemsa, or Wright–Leishman).

H. Siderotic granules (Pappenheimer bodies) (Figure 3.13K)
   1. *Siderotic granules* in Wright-stained RBCs are basophilic inclusions of ferritin or Fe-laden mitochondria. Siderotic granules may be difficult to differentiate from basophilic stippling on Wright-stained blood films, but they typically occur in focal clusters rather than as homogeneous stippling. They may be coarse and prominent, but they are most often fine and palely stained. Siderotic granules can be confirmed by positive staining for Fe.
   2. A *siderocyte* is an anucleate erythroid cell (reticulocyte or mature RBC) that contains one or more siderotic granules. If the siderotic granules are in a nRBC, the cell is a *sideroblast*; if the granules encircle the nucleus, the cell is a *ringed sideroblast*.
   3. Siderocytosis is commonly seen with regenerative anemias, perhaps related to increased Fe turnover of hemolytic disorders, and it occurs rarely secondary to certain toxicants (e.g., lead) and drugs (e.g., hydroxyzine, chloramphenicol in dogs). Siderocytosis and sideroblastosis may be related to whole-body or RBC mitochondrial Fe overload, and also have been reported as features of sideroblastic anemias, some myeloid neoplasms, and certain dyserythropoietic syndromes.[79]

I. Other: RBCs of adult camelids or crias may contain round to ovoid, pinkish to purplish, homogeneous to stippled or fragmented inclusions of undetermined cause and no apparent clinical significance (Figure 3.13L). They tend to be located centrally and have smooth, scalloped, or jagged borders.

VI. Abnormal RBC volume

A. RBCs appear two-dimensional on a Wright-stained blood film, and thus a cell's diameter is frequently considered to reflect its size. However, two RBCs with the same diameter but different thicknesses in a blood smear have different volumes. A RBC's

thickness is reflected by the cell's staining intensity. Thin cells (leptocytes) are lightly stained (more hypochromic), whereas thick cells are stained more intensely (more hyperchromic).

B. *Anisocytosis* has been defined as increased variation in the volumes of RBCs in analyzers or as increased variation of RBC diameters in blood films (loosely speaking "size"). Because spherocytes are globoid and not flattened out on a slide, they have decreased diameters and contribute to apparent (visual) anisocytosis even though their volumes are minimally or not decreased.
   1. Anisocytosis can be caused by macrocytes or microcytes mixing with each other or, more often, with normocytes. The diagnostic significance of anisocytosis depends on the cells that are creating the anisocytosis (see the next sections on macrocytes and microcytes). Anisocytosis is commonly associated with macrocytosis and thus regenerative anemias.
   2. RDW (see Analytical Principles and Method, Section II.F) is an automated reflection of anisocytosis based on volumes, not on the microscopic assessment of cell diameters.

C. A *macrocyte* is a RBC that has increased volume (Figures 3.9A and 3.14A).
   1. *Macrocytosis* is an increased concentration of macrocytes in blood, which can be reflected by a shift in the RBC cytogram or by an increased MCV. The concurrent presence of macrocytes with normocytes or microcytes yields anisocytosis, and the RDW may be increased.
   2. Macrocytosis is frequently seen with accelerated erythropoiesis because immature RBCs are typically larger than mature RBCs. In most species, these macrocytic reticulocytes are thought to undergo splenic remodeling to become normocytes.[6,8] In cats, however, nonreticulocyte macrocytes may persist for 6 wk or longer after a regenerative anemia, perhaps because of less splenic remodeling (see Physiologic Processes, Section I.C.2).[9] Macrocytes may also result from a skipped mitosis in nonneoplastic or neoplastic disorders with abnormal erythropoiesis.
   3. Poodles with hereditary poodle marrow dyscrasia (see Abnormal Morphologic Features of WBCs, Section VII.D, in Chapter 2) typically have uniform normochromic macrocytes (MCV: 80–110 fL).

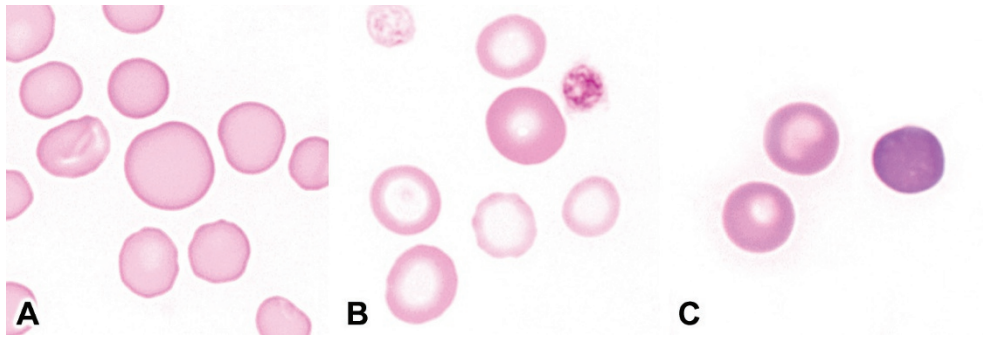


Figure 3.14. A. Macrocyte and resulting anisocytosis, horse. B. Leptocytic, hypochromic microcytes of Fe deficiency, dog. C. Normochromic and polychromatophilic microcytes, portosystemic shunt, dog.

D. A *microcyte* is a RBC that has decreased volume (Figure 3.14B and C).
   1. *Microcytosis* is an increased concentration of microcytes in blood, which can be reflected by a shift in the RBC cytogram or by a decreased MCV. The concurrent presence of normocytes or macrocytes yields anisocytosis; the RDW may be increased.
   2. Documented causes of microcytosis include Fe deficiency (concurrent hypochromasia is usually seen in dogs), hepatic failure or chronic hepatitis in dogs, canine hepatocellular carcinoma,[80] portosystemic shunts in dogs and cats, Fe sequestration in a minority of animals with chronic inflammation, and probably copper deficiency (see Anemia, Sections II.B.6 and 7). Microcytosis of unknown pathogenesis also has been reported in cats with feline infectious peritonitis (FIP), whether anemic or not,[81] and it might occur in some hyperthyroid cats, irrespective of anemia or erythrocytosis. In addition, microcytosis occurs with certain rare congenital RBC disorders including dyserythropoietic anemia and myopathy syndrome in English springer spaniels or Labrador retrievers[67,82] and some porphyrias.[83]
   3. As seen with Fe deficiency, microcytes are created by increased erythroid cell divisions that occur when the nRBC cellular Hgb concentration is not great enough to inhibit mitosis. A RBC can have a normal diameter microscopically but be a microcyte with increased central pallor because of its thinness and therefore decreased volume (a hypochromic microcyte and leptocyte).
   4. Some healthy dogs (e.g., some Akitas, shibas, Jindos, chow-chows, and shar-peis) have mildly to moderately lower MCVs than those of most breeds, similar to values in dogs with pathologic causes of microcytosis.[84] Young horses (about 1–6 mo of age) have lower MCVs (and MCHs) than mature horses.[85,86] Young kittens have lower MCVs than mature cats, and this has been associated with hypoferremia relative to adult concentrations, suggesting a transient Fe-deficient state during early growth.[87]
   5. Microscopically, spherocytes appear microcytic because of decreased diameters, but their volumes are typically WRI (they are ball-like cells rather than flat cells), and MCVs for the sample may be WRI or, when there is a regenerative response, increased.
   6. Sideroblastic anemias may be microcytic (see Other RBC Disorders, Section X).[79]

VII. Abnormal RBC shape (Table 3.8)
   A. A *poikilocyte* is any RBC with an abnormal shape, including all the shapes named in this section, as well as shapes for which there are no accepted labels (unclassifiable poikilocytes). *Poikilocytosis* is an increased concentration of poikilocytes in blood.
   B. Camelid RBCs are resistant to shape changes seen in other domestic mammals, perhaps related to the greater protein (e.g., band 3) content per unit area.[88,89]
   C. The significance of poikilocytosis depends on the number and type of poikilocyte(s). Unless a particular poikilocyte is present, on average, in every 100×-oil objective field, it is unlikely to be clinically significant unless there are low numbers of multiple poikilocytes that together suggest a disorder (e.g., schistocytes and keratocytes may suggest physical trauma to RBCs).

Table 3.8. Poikilocytes: features, clinical significance, and pathogeneses in domestic mammals (Figures 3.15 and 3.16)

Poikilocyte	Other name	Identifying features	Clinical significance	Pathogeneses
Acanthocyte (*acantho* = "spur")	Spur cell, burr cell[a]	1–20 irregularly spaced blunt or club-like projections (spurs)[b] of various lengths	Hemangiosarcoma, splenic infiltrative disease, hepatic or renal disorders	Unknown; some may form from changes in plasma and membrane lipids
Codocyte* (*codo* = "hat")	Target cell, Mexican hat (sombrero) cell	Central focus of Hgb that is surrounded by a ring of pallor that separates it from peripheral Hgb; may also be a leptocyte	Regenerative anemias; also seen with Fe deficiency and hepatic, renal, and lipid disorders	Excess membrane relative to Hgb content; may occur with membrane lipid changes
Dacryocyte (*dacyro* = "tear")	Spatulate cell in camelids	Teardrop to pear shaped	Camelids in health (few) or with Fe deficiency (many); some are artifacts	Unknown except artifacts caused by stretching during film preparation
Eccentrocyte (*eccentro* = "eccentric")	Bite cell, cross-bonded cell, hemighost	Eccentric dense-staining Hgb and adjacent clear space or crescent	Overwhelming exposure to oxidants; rare G6PD or FAD deficiencies	Fusion of membranes damaged by oxidants; Hgb forced to side
Echinocyte* (*echino* = "spiny")	Burr cell[a], crenated RBC	Vary from irregularly shaped cells (type I), to relatively short regularly spaced blunt (type II) or pointed (type III) projections	Hyponatremic dehydration, ATP depletion, certain venoms, toxins, and drugs; often artifacts	Multiple causes including prolonged exposure to alkaline glass while drying (see the text)
Elliptocyte	(See ovalocyte)	—	—	—
Keratocyte* (*kerato* = "horn")	Helmet cell	One or two projections (horns) adjacent to flattened cell margin; can include prekeratocytes with peripheral blister-like hole	Vasculitis, endocarditis, intravascular coagulation, hemangiosarcoma, caval syndrome	Most likely intravascular trauma; also occurs with Fe deficiency
Knizocyte	—	Appears to have a bar of Hgb between two regions of central pallor	Regenerative anemias, lipid disorders	Unclear; the RBCs are triconcave
Leptocyte (*lepto* = "thin")	—	Thin cell that appears as a hypochromic cell	Fe deficiency and potentially Fe restriction	Incomplete Hgb synthesis

Ovalocyte (*ovalo* = "egg")	Elliptocyte	Elliptical or oval cell	Band 4.1 deficiency, mutant spectrin, or myelofibrosis in dogs; Fe deficiency; idiopathic in cats	Abnormal membrane proteins in hereditary form, otherwise unknown
Pincered cell	Pincer or mushroom-like cell	Cell appears symmetrically pinched in on both sides	PK deficiency, intravascular trauma	Unknown
Pyknocyte (*pykno* = "condensed")	Irregularly contracted cell	Spheroid RBC with condensed or contracted Hgb and perhaps small tags of fragmented membrane	Overwhelming exposure to oxidants; rare G6PD or FAD deficiencies	Likely forms from eccentrocytes
Schistocyte* (*schisto* = "divided")	RBC fragment, schizocyte	Triangular, comma-shaped, round, or irregularly shaped portion of a RBC	Intravascular coagulation, vasculitis, endocarditis, hemangiosarcoma, caval syndrome	Same as keratocyte
Selenocyte (*seleno* = "moon")	Selenoid bodies	A damaged RBC that is crescent-shaped and has a large clear space	Associated with hemolytic anemias, fragile RBCs	Artifact (see the text)
Spherocyte* (*sphero* = "round")	—	Decreased central pallor, decreased cell diameter, increased staining intensity, and smooth margins	Immune hemolysis,* ATP depletion, some venoms, clostridial α-toxin, band 3 deficiency, fragmentation, dyserythropoiesis, zinc toxicosis	Membrane loss or damage, RBC fragmentation, abnormal cytoskeleton
Stomatocyte (*stomato* = "mouth")	—	Elongated (slitlike or mouthlike) area of cytoplasmic pallor	Young RBCs or hereditary stomatocytosis of dogs	Folding of excess membrane
Torocyte (*toro* = "doughnut shaped")	—	Punched-out, central clear space that creates a doughnut-shaped cell	None; do not confuse with hypochromasia	Artifact

* A relatively common poikilocyte

[a] Classifying cells as burr cells is not recommended because the name is used for acanthocytes and echinocytes.

[b] Criteria for classifying RBCs as acanthocytes vary; cells with more projections that are less club-like may occur in other conditions.

D. Poikilocytes can be artifacts or reflect a physiologic state.
   1. Artifactual shape changes may occur with in vitro aging, smearing trauma, contact with glass, or smear drying; most of these poikilocytes may also be pathologic. Additionally, when bovine blood was heated to about 50–55 °C, marked artifactual poikilocytosis occurred and was characterized by budding fragmentation and elongated and irregularly contoured RBCs, some similar to dacryocytes.[90]
   2. Neonatal calves can have a marked increase in spiculated RBCs with features of acanthocytes, echinocytes, or schistocytes. The pathogenesis of the changes is not established but may be related to the presence of a unique Hgb molecule or its interactions with RBC membrane proteins.[91] Fe deficiency may contribute to anemia and poikilocytosis.[91,92]

E. Poikilocytes are defined by their microscopic features, and specific terms should be used whenever possible (Table 3.8). There are differences among veterinary laboratory professionals and clinical pathology resources regarding identification, naming, semiquantitation, and interpretation of poikilocytes.

F. Acanthocyte (spur cell) (Figures 3.15A and 3.16F)
   1. *Acanthocytes*, as described by Bessis, are RBCs with relatively few irregularly spaced projections of various lengths, appearing distinctly different from echinocytes.[93] These classically shaped acanthocytes are most common in dogs, and the projections may bud off to form microspherocytes. They have been associated with splenic and hepatic disorders, including hemangiosarcoma in dogs[94] and hepatic lipidosis in cats.[95] With hepatic disorders, changes in plasma lipids may alter RBC membrane lipids to cause acanthocyte formation just as acanthocytic change in people is often associated with abnormal lipid composition (high cholesterol to phospholipid ratio) acquired within a RBC's membrane during circulation. Some dogs fed a cholesterol-rich diet developed cholesterol-enriched RBC membranes, RBCs "resembling acanthocytes or spur cells," and even anemia with mild reticulocytosis, supporting hemolysis; these changes reversed with return to a normal diet.[96] Infiltrative splenic disease may decrease splenic function and clearance of acanthocytes; in people, acanthocytosis has been reported after splenectomy.
   2. There is little or no standardization of the definition or distinction between acanthocytes and echinocytes in current practice, and many poikilocytes that do not meet the Bessis definition of acanthocytes are reported as acanthocytes; the terms acanthocyte-like and irregularly spiculated cells have also been proposed for such cells.[97] The cause and significance of many acanthocytoid cells is unclear, and they have been associated with a wide variety of neoplastic and nonneoplastic conditions.
   3. Acanthocytes or acanthocytoid cells have been seen concurrently with keratocytes and schistocytes in cases of lymphoma, hemangiosarcoma, cirrhosis, pancreatitis, intravascular coagulopathy, ventricular outflow obstruction, and glomerulonephritis. The concurrent finding of acanthocyte-like cells, keratocytes, and schistocytes suggests that some acanthocytoid cells may form by intravascular trauma,[97,98] but concurrent mechanisms may be at play given that keratocytosis and schistocytosis occur without acanthocytosis and vice versa.

G. Blister cell (see eccentrocyte and keratocyte in the following Sections L and O)

H. *Burr cell* is a name that has been applied to several types of spiculated RBCs, but usually echinocytes.

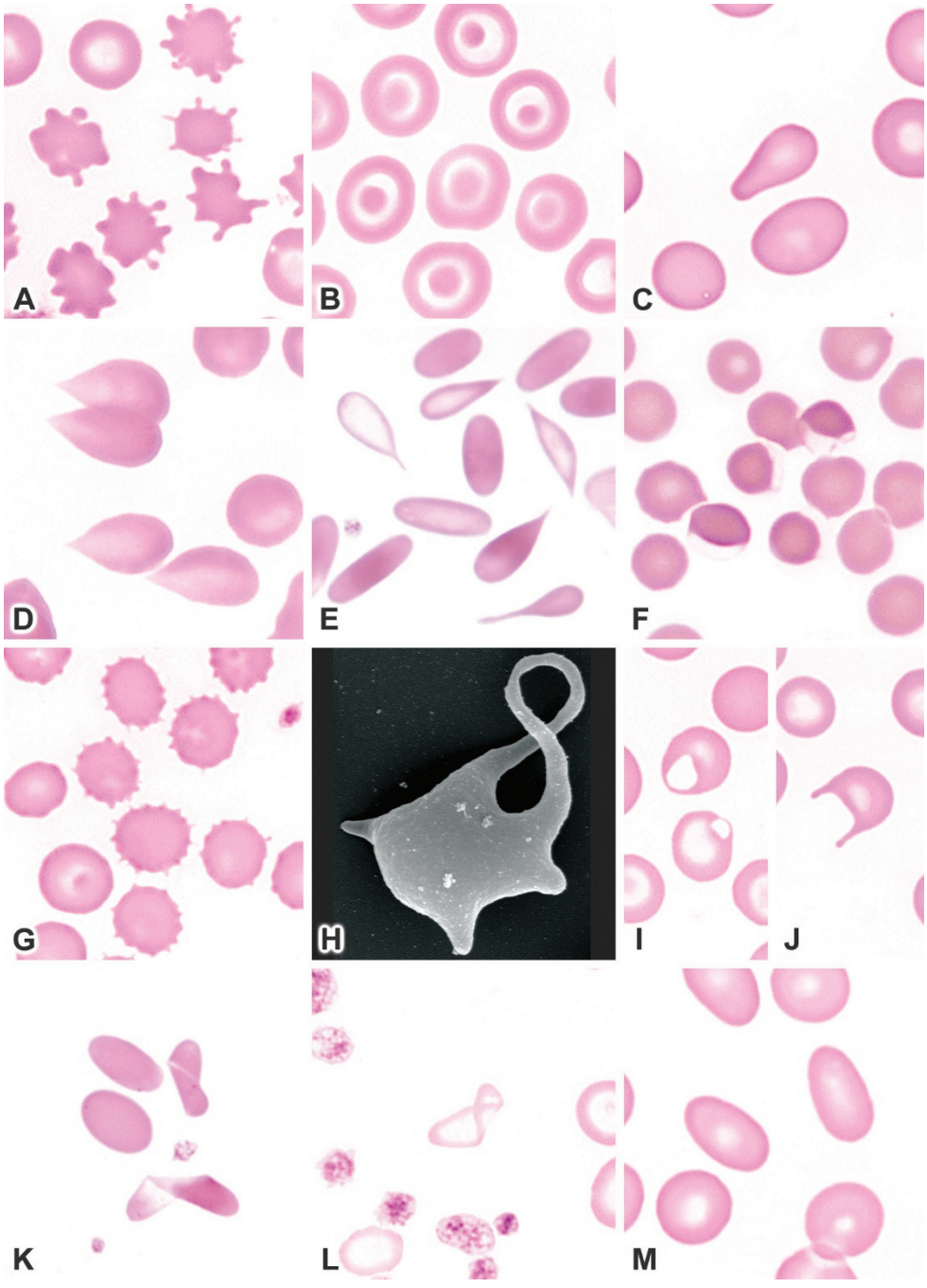


Figure 3.15. Poikilocytes (part 1). A. Acanthocytes, splenic hemangiosarcoma with hepatic metastases, dog. B. Codocytes, dog. C. Dacryocyte, dog. D. Artifactual dacryocytes, dog. E. Normochromic and hypochromic dacryocytes (spatulate cells) and a spindle-shaped cell, alpaca. F. Eccentrocytes, horse. G. Echinocytes, dog. H. Prekeratocyte, scanning electron micrograph, cat. I. Prekeratocytes, dog. J. Keratocyte, dog. K. Folded alpaca RBCs. L. Folded hypochromic, microcytic leptocyte of Fe deficiency, dog. M. Ovalocytes (elliptocytes), dog.

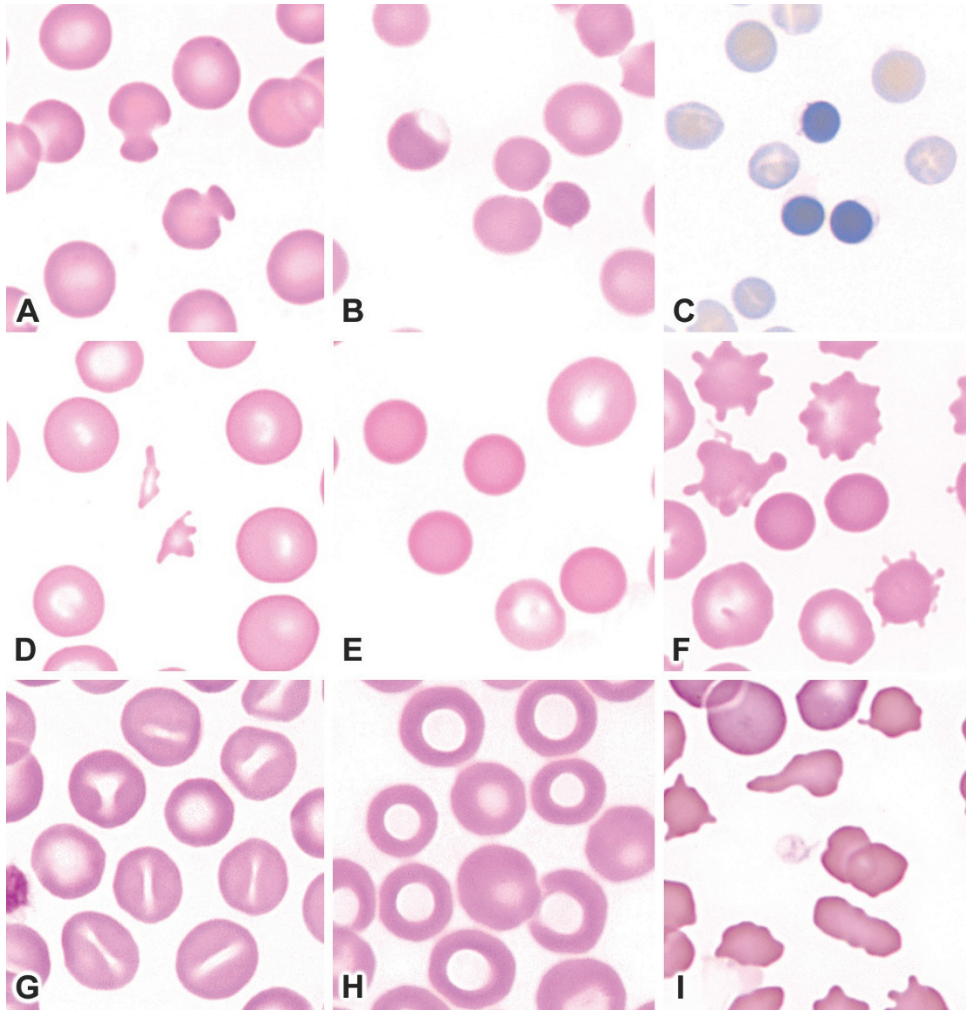


Figure 3.16. Poikilocytes (part 2). A. Pincered (mushroom) cells, dog. B. Pyknocyte (4 o'clock) with slightly irregular shape, and an eccentrocyte (*left of center*), horse. C. Pyknocytes, NMB vital stain, horse. D. Schistocytes, dog. E. Spherocytes, IMHA, dog. F. Acanthocytes and fragmentation-derived microspherocytes, dog. G. Stomatocytes, dog (from ASVCP slide contributed by Dr. D.E. Brown, et al., 1992). H. Torocytes (artifact), dog. I. Unclassifiable poikilocyte (looks like crooked finger or boot), cat.

I. Codocyte (target cell, Mexican hat cell, sombrero cell) (Figure 3.15B)
   1. A *codocyte* is a target-like RBC with a peripheral ring and central "bull's eye" of Hgb separated by a nonstaining ring. This shape results from a central bulge in the cell caused by an increased ratio of cell membrane to cell volume.
   2. Codocytosis is commonly seen in regenerative anemias. This is because young RBCs have excess membrane and a decreased cellular Hgb concentration (not mass). When not associated with a regenerative anemia, codocytosis is seen in hypochromic states (e.g., Fe deficiency) and when RBCs have excess membrane (e.g., hepatic, renal, and lipid metabolism disorders). They have been associated with diabetic hepatopathy and diabetic ketoacidosis in dogs.[99]

J. Crenated RBCs (see echinocyte in the following Section M)
K. Dacryocyte (Figure 3.15C–E)
   1. *Dacryocytes* are teardrop- or pear-shaped RBCs. Dacryocytosis has been reported in some animals with marrow diseases such as myelofibrosis and neoplasia, but ovalocytosis, not dacryocytosis, was associated with myelofibrosis in a study of myelofibrosis in dogs.[100]
   2. In camelids, ovalocytes with a point or short stalk at one end have been called dacryocytes or *spatulate cells*. Such cells can be seen in health, but they can be increased along with spindle-shaped RBCs in llamas and alpacas with Fe-deficiency anemias.
   3. Artifactual dacryocytes may form because of RBC stretching during blood film preparation. Artifactual dacryocytes tend to have sharp points, occur in streaks, and tend to point in the same direction because directional forces of the slide preparation create them.
   4. Some people apply the term dacryocyte to cells that others would interpret as one-horned keratocytes.
L. Eccentrocyte (bite cell, cross-bonded cell, or hemighost cell) (Figure 3.15F)
   1. *Eccentrocytes* are cells formed when membrane oxidation leads to a bonding of RBC membranes to form a peripheral, crescent-shaped region of the cell (sometimes called a blister); the cell's Hgb is displaced eccentrically where it forms a spheroid mass that may have increased staining intensity like a spherocyte. The Hgb in hypochromic RBCs of Fe-deficient camelids may be distributed to one side of RBCs, but such cells are not eccentrocytes.
   2. Membranes are damaged by some of the same endogenous or exogenous oxidants that cause Heinz body anemias. Eccentrocytes may also form when reducing pathways in RBCs are defective, e.g., in G6PD-deficient[101] or FAD-deficient[102] horses (rare conditions).
   3. If enough RBCs in a blood sample are eccentrocytes, the sample's CHCM and MCHC may be truly increased (same mass of Hgb in less volume).
M. Echinocyte (burr cell, crenated cell) (Figure 3.15G)
   1. *Echinocytes* are RBCs with relatively short and relatively regularly spaced pointed or blunt projections. The number and shape of spicules classify echinocytes. Type I echinocytes are irregular or angular cells lacking distinct spicules, type II echinocytes have multiple regularly spaced blunt spicules, and type III echinocytes have multiple regularly spaced sharp projections.[103,104] This classification is similar to stage 1, stage 2, and stage 3 echinocytic changes seen with electron microscopy.[105]
   2. Echinocytes are thought to form when the surface area of the outer lipid layer of the cell membrane increases relative to that of the inner lipid layer because of insertion of lipids or amphipathic drugs. They may also form secondary to increases in pH, RBC ATP depletion, damage by phospholipases, and cellular dehydration. Consequently, pathologic echinocytosis has been associated with several disorders:
      a. RBC dehydration (especially with hyponatremia and hypochloremia in horses)[103,106]
      b. Strenuous exercise (in racing horses)[104]
      c. Doxorubicin toxicosis[107]
      d. Reaction to anionic drugs such as phenothiazine[108]
      e. PK deficiency (echinocytes or spheroechinocytes seen in some dogs)[109,110]
      f. Rattlesnake, coral snake, or bee envenomation[111–113]
      g. Hemolytic anemia caused by clostridial infection in a horse[114]

3. Echinocytes commonly form artifactually after blood is collected and while the blood film is drying, thus reducing the diagnostic value of echinocytosis. The membrane changes are probably due to the alkalinity of glass.[115] RBCs from some sick animals may be prone to crenation (forming a scalloped or notched membrane), and crenation may increase with storage due to RBC ATP depletion or increased plasma lysolecithin formation.[115]
4. Differentiation of artifactual and pathologic echinocytes can be difficult and is not often pursued. These three methods might assist with the challenge:
   a. Make another blood film and use a hair drier or other means to dry the blood quickly.
   b. Examine blood films prepared on plastic slides or coverslips. Artifactual crenation is less likely to occur on plastic.
   c. Examine RBCs in a wet mount of blood on a slide. If echinocytes are not present, those seen in the stained film can be considered artifacts.

N. Elliptocyte (see ovalocyte in the following Section R)
1. An *elliptocyte* is a RBC with a shape approximating that of an ellipse. It is the typical shape of mature RBCs in camelids. In other mammalian species, elliptocytosis (ovalocytosis) is caused by acquired or congenital disorders.
2. Ovalocytosis and elliptocytosis are typically considered synonyms. Some prefer one term over the other, using ovalocyte for plumper egg-shaped cells and elliptocyte for cells that more resemble the actual geometric shape of an ellipse.

O. Keratocyte (helmet cell) (Figure 3.15H–J)
1. *Keratocytes* (not keratinocytes, which are keratin-containing squamous epithelial cells) are RBCs with one or two projections (horns) from one or more membrane sites where a hole had formed through the cell; the cell is usually flattened adjacent to the horn(s) (Figure 3.15J). Keratocytosis may be caused by trauma to RBCs within the vascular system; the same processes may create schistocytes (see Schistocyte for causes). Keratocytes and schistocytes commonly form in Fe deficiency, likely because the cells are more fragile and less deformable, in which case other RBC abnormalities supportive of Fe deficiency would be present. Keratocytes have also been reported in feline hepatic disease[95] and doxorubicin toxicity in cats.
2. An intermediate form sometimes called a *prekeratocyte* (blister cell) has a cytoplasmic clear space (blister) caused by a hole through the cell (Figure 3.15H and I). Intravascular trauma is thought to form these cells, with subsequent breakage of the cytoplasmic arc enclosing the hole yielding one- or two-horned keratocytes. Prekeratocytes and keratocytes are commonly reported together as keratocytes.
3. Some forms of keratocytes, particularly those with one horn, have been classified as budding fragmentation, acanthocytes, or dacryocytes.

P. Knizocyte
1. A *knizocyte* is a triconcave RBC as seen by scanning electron microscopy, but by routine light microscopy, it appears to have a bar of Hgb crossing the RBC between two regions of pallor.
2. Though rarely reported, knizocytes may be increased with regenerative anemias, stomatocytosis, and lipid disorders, including those associated with hepatic disease.

Q. Leptocyte (Figures 3.14B and 3.15K and L)
1. A *leptocyte* is a thin cell. Some codocytes and most hypochromic RBCs are leptocytes (see hypochromic RBCs and codocytes in Morphologic Features of RBCs, Sections III.C and VII.I). Camelid RBCs are thin and therefore can be considered

leptocytes. Thin cells can fold on themselves. With Fe deficiency, RBCs may become thinner and more prone to folding.

2. Some people consider leptocyte a synonym for codocyte or a term for a hypochromic RBC. However, while codocytes and hypochromic RBCs can be leptocytes, not all leptocytes are codocytes or hypochromic cells. Also, immature RBCs are not leptocytes (thin cells), but they are commonly codocytes and hypochromic based on MCHC and CHCM.

R. Ovalocyte (elliptocyte) (Figure 3.15M)

1. An *ovalocyte* (elliptocyte) is an ovoid or elliptical RBC. Camelid, avian, reptilian, and amphibian RBCs are ovalocytes in health, with round cells being abnormal or immature in these species. Ovalocytosis is pathologic in other species. Acquired ovalocytosis is seen in dogs with myelofibrosis[100,116] and in animals with Fe-deficiency anemias (along with other abnormal RBC features). Ovalocytes may also occur with portosystemic shunts[117] and doxorubicin toxicity.[118]
2. RBCs in some ill cats assume an irregular, elongated form that may have a scalloped margin and/or broad appendage (Figure 3.16I).[119] These poikilocytes have been reported as ovalocytes, elliptocytes, acanthocytes, keratocytes, or unclassifiable poikilocytes. Their pathogenesis is unknown. More-typical elliptocytes have been noted in cats that have hepatic disease, including hepatic lipidosis.[95]
3. Two types of canine hereditary ovalocytosis (elliptocytosis) have been reported, one associated with protein band 4.1 deficiency[120] and one with mutant membrane spectrin.[121] Clinical aspects of these disorders are presented in this chapter (Other RBC Disorders, Sections VII and VIII).
4. Elliptocytes (ovalocytes) have been subclassified: Type I is nearly circular, type II is oval, and type III is more elongated.[115]

S. Pincered (pincer, mushroom) cell (Figure 3.16A)

1. Pincered cells look like they have been pinched together, and they may resemble mushrooms. They are rare cells and rarely reported in veterinary species, but they have been associated with RBC trauma and PK deficiency in a Cairn terrier (unpublished case report).
2. In people, pincered cells have been associated with a variety of disorders including RBC fragmentation, hereditary spherocytosis, and erythroleukemia.

T. Pyknocyte (irregularly contracted cell) (Figure 3.16B and C)

1. A *pyknocyte* is a spheroid cell that typically has a slightly irregular contour because of one or more membrane tags seen clearly by electron microscopy.[101] Pyknocytosis is associated with eccentrocytosis, and pyknocytes likely form from eccentrocytes, though oxidative damage might cause both directly. With serial evaluation, eccentrocytosis may be prominent early after exposure to an oxidative toxicant and pyknocytosis may predominate later.
2. Via light microscopy, some pyknocytes look like spherocytes. However, these spheroid pyknocytes are usually accompanied by eccentrocytes and pyknocytes with membrane tags, so the pathologic changes can be recognized.
3. At least in horses, pyknocytes stain more intensely with vital NMB staining than do discocytes or spherocytes.

U. Schistocyte (schizocyte or RBC fragment) (Figure 3.16D)

1. A *schistocyte* is a RBC fragment. Although often irregular in shape, schistocytes may be round (microspherocytes). Schistocytosis occurs when rigid structures or rheologic forces traumatize RBCs. Schistocytes may be hypochromic (pale) if they lost sufficient

Hgb at the time of fragmentation or if they formed from hypochromic cells in animals with Fe deficiency.

2. Pathologic states associated with schistocytosis include intravascular coagulation, glomerulonephritis and hemolytic uremic syndrome, other vasculitides, hemangiosarcoma, caval syndrome of dirofilariasis, endocarditis, hepatic disease, cardiac disease (valvular disease, aortic or pulmonic stenosis, septal defects), and Fe deficiency. Schistocytes may become prominent after splenectomy, along with other poikilocytes,[122] and they are described in people with severe burns or one of several congenital RBC disorders. They commonly form in Fe deficiency.

V. Selenocyte (selenoid body)

1. Selenocytes are not a commonly recognized poikilocyte in domestic mammals but have been found in anemic and nonanemic animals (authors' observations).
2. Their formation is described as a two-step process: (i) a hole is formed in the RBC membrane, and (ii) the hole becomes much larger while the cell is spreading on the slide; the remaining RBC appears as a large, lightly colored crescent.[115] RBCs in lipemic blood may be more prone to selenocyte formation.[123] Author experience suggests that quick dip staining may promote their formation.

W. Spherocyte (Figure 3.16E and F)

1. A *spherocyte* is a spherical RBC. Among domestic mammals, spherocytes are most easily recognized in blood films of canine blood because RBCs of most dogs have enough central pallor to contrast clearly with the spherocytes that lack central pallor. With careful evaluation, however, trained microscopists can sometimes recognize or suspect spherocytes in other species.
2. A moderate to marked spherocytosis in dogs is usually associated with IMHA; a mild spherocytosis may or may not be. In IMHA, the spherical shape is thought to result from the loss of RBC membrane without a corresponding loss of RBC cytoplasm. These cells cannot flatten on a slide, so their diameters are decreased, and they appear microcytic. Because they are thick, they stain more intensely. However, their volumes are typically not altered appreciably.
3. Spherocytes may also form from acanthocytes through budding fragmentation, in which case they have lost cytoplasm and have become smaller. Other microspherocytes form with other fragmentation-induced poikilocytes in fragmentation anemias. Spherocytes or spheroechinocytes are reported to occur with ATP depletion of RBCs, e.g., in PK-deficient dogs[124] and cows with hypophosphatemic hemoglobinuria,[125] in bee-sting anemias,[113] with some snake envenomations,[111,126,127] in hereditary band 3 deficiency in Japanese black cattle,[128] and with other findings in dyserythropoietic anemia and myopathy syndrome of English springer spaniels.[66] They are also reported in some dogs with zinc toxicosis, in which case they must be differentiated from pyknocytes; zinc might promote band 3 clustering and binding of autologous senescence antibodies for immune-mediated clearance, thus potentially causing spherocyte formation.[129]
4. Spherocytosis may be falsely detected if one assesses cells too near the feathered edge of a smear where artifactual distortion of RBCs makes RBCs lack central pallor; however, these RBCs typically do not appear smaller and darker as spherocytes should. Pyknocytes have been falsely identified as spherocytes.
5. Processes that produce spherocytes may also produce spheroid cells that are not perfect spheres and have small amounts of central pallor from single cup-like indentations. These cells have been called stomatospherocytes.

X. Spindle cells (fusiform or spindle-shaped RBCs) (Figures 3.10C, 3.13L, and 3.15E)
   1. A RBC referred to as a *spindle* cell is a RBC forming points at opposite ends of the cell.
   2. These doubly pointed cells are most common in camelids, few being present in health. Many may be present with anemia, notably Fe-deficiency anemia, in which case singly pointed (spatulate or dacryocytic) cells may also be common.

Y. Stomatocyte (Figure 3.16G)
   1. *Stomatocytes* are cupped RBCs, and the bowl- or cup-shaped invagination may be of different depths and round to elongate, the latter forming an elongated area of pallor (a slit or stoma) instead of a circular area of central pallor. Young RBCs (polychromatophilic RBCs or young macrocytes) frequently are stomatocytes, although this may not be noted in CBC reports.
   2. Stomatocytosis also can be caused by a hereditary defect in the RBC membrane: hereditary stomatocytosis of Alaskan malamutes with concurrent chondrodysplasia, Drentse patrijshonds with concurrent hypertrophic gastritis,[130] miniature schnauzers (asymptomatic),[131] standard schnauzers (asymptomatic),[132] and Pomeranians[122] (see Other RBC Disorders, Section V).

Z. Torocyte (Figure 3.16H)
   1. A *torocyte* is a RBC with a sharply punched out center within a ring of deeply stained Hgb. It represents an artifactual shape change.
   2. Torocytes should not be confused with hypochromic cells that have marked central pallor. In hypochromic cells, the peripheral Hgb is typically paler and fades into the central pallor rather than abruptly stopping as in a torocyte.

ANEMIA

I. General information
   A. Anemia can be defined as a decreased [RBC], a decreased blood [Hgb], or a decreased Hct. These generally change proportionately because they are all assessments of the RBC content of blood. However, they may not be uniformly decreased, because of variations in RIs or the presence of abnormal RBCs, i.e., an abnormal RBC volume or intracellular Hgb concentration.
   B. Anemia caused by an increase in plasma volume rather than a decrease in the volume of circulating RBCs has been called *dilutional anemia* or *relative anemia*. It may be caused by fluid administration or fluid retention. Fluid retention can contribute to anemia in states that are physiologic (pregnancy) or pathologic (e.g., heart failure, cirrhosis, renal failure). Similarly, anemia may be masked by hypovolemia (e.g., dehydration). A decreased Hct caused by splenic pooling of RBCs (e.g., anesthesia) would also be a relative anemia but not a dilutional anemia. Some definitions restrict anemia to decreases in total-body RBC volume or mass, or so-called *absolute or true anemias*; however, total RBC mass is not easily determined. In this book, decreases in [RBC], [Hgb], or Hct are considered to reflect anemia, causes of which may include hemodilution or splenic pooling of RBCs.
   C. Recognition of anemia relies on use of appropriate RIs for Hct, [Hgb], or [RBC], which can vary with breed, age, and lactation.
      1. Breed: These RIs are higher for greyhounds, Afghan hounds, salukis, and whippets, so values reflecting health in other dog breeds may reflect anemia in these breeds.[133] Similarly, the lower reference limits for [Hgb] and Hct in Maine coon cats appear to be substantially greater than in typical cat breeds.[134] In equids, RIs for [RBC], [Hgb], and

Hct may be as much as 25 % lower in draft horses, ponies, and donkeys compared to Arabs, thoroughbreds, standardbreds, Appaloosas, and quarter horses.

2. Age: After the first several days of life, these values may be mildly to moderately lower than adult values for several months in dogs.[23,135] In other species, they may similarly dip below adult values in the first few months of life and remain near the lower reference limits for adults while the young animals are still growing rapidly.
3. Regarding cattle, these values are expected to be lower in lactating cows than in nonlactating cows, in beef cows than in dairy cows, and in bulls than in cows.[136] These differences should be considered when interpreting data, but specific RIs are typically not available for each group.

D. Anemia is a state or diagnostic problem rather than a disease. Its major significance is a reduced capacity of blood to transport O_2 to tissues. Anemia unrelated to dilution or splenic pooling develops when there is one or more of the following:
1. Increased RBC loss due to blood loss
2. Accelerated RBC destruction (pathologic hemolysis)
3. Decreased effective RBC production

E. Clinical signs caused by anemia reflect decreased O_2-carrying capacity and include decreased exercise tolerance, weakness, depression, and rapid respiration (tachypnea).

F. The major physical examination finding is pale mucous membranes (gingival, conjunctival, or vulvar) due to decreased RBC content in capillaries. With marked anemia, blood becomes less viscous and may cause a systolic heart murmur.

II. Classifications of anemias

There are three common classification systems, each with its advantages and limitations in certain clinical situations. The end goal is to classify anemias by specific cause, but other classifications promote accurate communication and allow focusing of differentials.

A. Classification by marrow responsiveness
1. This classification system is primarily based on the presence or absence of reticulocytosis in blood, but other blood and marrow findings may influence the classification. Reticulocyte assessment is not useful in equids.
   a. *Regenerative anemia* is anemia with a concurrent reticulocytosis or progressive Hct increases, the latter being particularly important for resolving anemias and for anemia in horses. Marrow may be responsive prior to a reticulocytosis (preregenerative anemia). Until there is a reticulocytosis (or progressive Hct increases), one cannot be certain that erythropoiesis is effective and that the anemia is regenerative.
   b. *Nonregenerative anemia* is anemia without concurrent reticulocytosis or a progressive increase in Hct. It may prove to have been preregenerative.
   c. Reticulocytosis may occur in the absence of anemia (see Reticulocytosis without Anemia).
2. Reticulocytosis is typically established by finding an increased RC, increased CRP, or increased polychromasia (see Analytical Principles and Methods, Sections II.H and III.K–N, and Morphologic Features of RBCs, Section III.D). Use of breed-specific RIs has been suggested for certain cats (e.g., Maine coon cats, Siberian cats) based on comparisons to published breed-nonselective values, but concurrent breed-nonselective data were not generated, and for the Maine coon cats, four potential outliers were included that extended the proposed upper reference limit for RC to more than three times the median (250,000/μL versus 73,000/μL).[134,137]

a. Documenting reticulocytosis is the most reliable, single routine method of establishing accelerated erythropoiesis (except in horses).
b. In most species, reticulocytosis is expected by ~3–4 d after Epo stimulates marrow. Peak production is expected about 7–10 d after stimulation. The reticulocyte response in cats may be different.[19,21] In a study of five cats after single episodes of blood loss, the aggregate reticulocytosis peaked 4 d later and then diminished to pre–blood loss concentration by day 9. The punctate reticulocytosis peaked 9 d after blood loss and did not return to baseline until after day 21.[19]
c. Horses very rarely release polychromatophilic RBCs from marrow, so assessment of polychromasia is not useful in horses. Reticulocyte enumeration is also not very useful in horses.
   (1) However, horses with an erythropoietic stimulus may release reticulocytes that are detectable by automated analyzers (e.g., ADVIA 120): RCs were 5–10 × 10^3/μL after phlebotomy induced anemia in horses,[26] the RC was 57 × 10^3/μL in a horse with a hemolytic anemia,[114] and it was 82,000/μL (automated) and 60,000/μL (microscopic) in a foal with NI.[25]
   (2) The presence of macrocytes in equine blood suggests, but does not prove, marrow responsiveness to Epo in most clinical situations.
   (3) Bone marrow examination may provide evidence for marrow Epo responsiveness in horses (decreased myeloid to erythroid ratio or increased marrow RP), but it is rarely done for this purpose.
d. Splenic contraction related to excitement or exercise shortly before or at the time of blood collection may transiently increase RP and RC.[138]

3. Spurious increases in RP and RC can be great enough to cause pseudoreticulocytosis (spurious reticulocytosis).
   a. Rarely, mild to marked pseudoreticulocytosis has been reported in dogs with acute or chronic leukemia.[139,140] Small neoplastic cells or cytoplasmic fragments of neoplastic cells may be detected as young, high-fluorescence reticulocytes, yielding a discontinuous reticulocyte scatter rather than the continuous scatter expected with a regenerative response (Sysmex analyzer).
   b. Parasitemia caused by large *Babesia* did not cause reticulocytosis but falsely increased the RP and RC determined by polymethine staining (ProCyte Dx and Sysmex analyzers), yielding a discontinuous reticulocyte cluster of mostly low-fluorescence cells.[141] False increases were supported by the absence of polychromasia and much lower values with NMB staining (microscopic method, LaserCyte analyzer).
   c. Other potential causes of spurious RP and RC results depend on the method (stain) and may include many Heinz bodies, giant platelets, platelet clumps, Howell–Jolly bodies, siderotic inclusions, or porphyria (autofluorescent RBCs). Disproportionate omission of mature RBCs from RBC analysis can falsely increase the RP and RC, e.g., with RBC agglutination or microcytosis.
   d. Additionally, unexplained false increases may occur with storage of canine[52] or feline[142] samples.
   e. The term *pseudoreticulocytosis* has also been applied to the reticulocytosis seen with delayed RNA degradation of abnormal RBCs (see Analytical Principles and Methods, II.N.3).

4. The following RBC abnormalities are common in regenerative anemias, but each may also be found in nonregenerative anemia (or even the absence of anemia), so they do not

alone indicate regeneration: macrocytic and/or hypochromic indices, anisocytosis and increased RDW, Howell–Jolly bodies, rubricytosis, codocytosis, or basophilic stippling.

5. Mild to moderate bone marrow erythroid hyperplasia with a left-shifted erythroid lineage may reflect a pending regenerative anemia in an animal without reticulocytosis. Marked erythroid hyperplasia with a moderate to severe anemia and without reticulocytosis is usually caused by ineffective erythropoiesis, e.g., PIMA. If there is a mild anemia, erythroid hyperplasia could represent the later stages of a resolving anemia when there is effective erythropoiesis but less Epo stimulus; reticulocytes are not released enough to cause reticulocytosis, but RBCs are released enough to increase the Hct.
6. Regenerative anemia (responsive anemia)
   a. A regenerative status indicates that a bone marrow is regenerating a replacement population of RBCs. This occurs primarily in response to blood loss or hemolysis. It is rarely associated with erythroid neoplasia in cats. It may occur with resolution of some causes of nonregenerative anemia.
   b. Regeneration may be blunted (diminished and/or delayed) by concurrent conditions associated with nonregenerative anemias; these include inflammation, severe anorexia, and food deprivation.[143]
7. Nonregenerative anemia (nonresponsive anemia)
   a. This occurs because of disorders that directly or indirectly cause defective or reduced RBC production. During the first few days after hemolysis or blood loss, an anemia will be classified as nonregenerative because the marrow has not had time to produce a reticulocytosis. A persistent nonregenerative status indicates that bone marrow is not sufficiently regenerating a replacement population of RBCs.
   b. Potential bone marrow findings include erythroid hypoplasia, marrow aplasia, red cell aplasia, myelofibrosis, myelophthisis, a hyporesponsive erythroid lineage (not clearly hypoplastic), mild to moderate erythroid hyperplasia in early responsive anemias, or erythroid hyperplasia with or without maturation arrest in conditions of ineffective erythropoiesis.
   c. Most nonregenerative anemias are normocytic normochromic anemias without poikilocytosis or other RBC abnormalities. However, blood may contain the following RBC abnormalities related to the underlying disease process: Howell–Jolly bodies, rubricytosis with or without dysplasia, codocytosis, basophilic stippling, macrocytes or microcytes, hypochromic RBCs, ovalocytes, or other poikilocytes.

B. Classification by RBC indices (morphologic classification)
   1. This classification system is based on MCV and MCHC (or CHCM) (Table 3.9). Classification should be further characterized by examining RBCs on a Wright-stained blood film. In the original classification system, there was not a category for blood samples with increased MCHC values because such values were considered to be erroneous, and they usually are. However, an increased MCHC or CHCM may be valid and reflect a rare pathologic state, so they are included here. Although the MCV and MCHC (CHCM) – not MCH! – are used to characterize RBCs in anemic animals, abnormal values can be found when anemia is not present. In such samples, they are frequently related to dyserythropoiesis or in vitro artifacts.
   2. General concepts
      a. The value of this classification is limited by several factors:
         (1) MCV and MCHC are inaccurate in aged samples; MCV increases and MCHC decreases, yielding falsely macrocytic and/or hypochromic populations or

Table 3.9. Causes of anemias classified by RBC indices (MCV and MCHC or CHCM)

Anemia classification	MCV	MCHC[a]	Disorders or conditions that cause the anemia
Normocytic normochromic	WRI	WRI	If persistent, then commonly disorders that reduce erythropoiesis; most anemias are initially normocytic normochromic; may be regenerative
Macrocytic hypochromic	↑	↓	Regenerative response to blood loss or hemolysis; some dogs with PIMA and myelofibrosis or hereditary stomatocytosis; hydroxyurea; in vitro cell swelling[b] with other cause of anemia
Macrocytic normochromic	↑	WRI	Regenerative response to blood loss or hemolysis; defective erythropoiesis (FeLV induced, poodle macrocytosis, other); RBC agglutination; in vitro cell swelling[b] with other cause of anemia
Microcytic[c] hypochromic	↓	↓	Fe and possibly B_6 or Cu deficiency; some chronic hepatopathies or portosystemic shunts; chronic inflammation; dyserythropoietic anemia and myopathy syndrome in Labrador retrievers; erythropoietic porphyria
Microcytic[c] normochromic	↓	WRI	Fe deficiency; some chronic hepatopathies or portosystemic shunts;[b] dyserythropoietic anemia and myopathy syndrome in English springer spaniels or Labrador retrievers; chronic inflammation
Normocytic hypochromic	WRI	↓	Possibly any cause of hypochromic cells but without enough change in MCV for it to be high or low (MCV close to the upper or lower limit of the RI)
Normocytic hyperchromic[c] Macrocytic hyperchromic[c] Microcytic hyperchromic[c]	WRI ↑ ↓	↑ ↑ ↑	MCHC may be falsely increased (check CHCM) and MCV falsely decreased with RBC shrinkage; marked eccentrocytosis and pyknocytosis (rare)

[a] CHCM may be used in place of MCHC, which is more prone to artifactual increases.
[b] See the text for information about artifactual changes in RBC indices.
[c] Microcytic and hypochromic cells may be present but unrelated to anemia in cats with FIP or hyperthyroidism, in dogs with breed-related microcytosis, or with in vitro cell shrinkage.[b]
[d] The *hyperchromic* classification was not part of the original morphologic classification system because increased MCHC values were considered to be erroneous and usually are. However, the increased MCHC or CHCM rarely may represent a pathologic state and thus we have added *hyperchromic* to the system.
Note: RBC concentrations in juvenile animals typically are lower than in mature animals, and young, growing animals may have lower MCV values than adults (see the text). Appropriate RIs must be used. Classification by RBC indices is most useful when testing samples promptly, before significant in vitro changes.

masking abnormal indices (microcytic cells may appear to be normocytic). Such changes occur within hours and can alter classification by 12 h.
(2) Inaccuracies occur with sufficient interference from lipemia, agglutination, or Heinz bodies.
(3) Results may also be significantly affected by changes in patient plasma osmolality and by differences in osmolality of diluting buffers used in hematology analyzers.

b. Because the indices are mean values for the entire population, they are relatively insensitive to mild or early changes. RBC cytograms or blood film examinations are typically more sensitive methods of detecting macrocytic, microcytic, or hypochromic cells. With either method, it is possible to have a normocytic normochromic anemia with detectable and significant macrocytic, microcytic, or hypochromic populations.

c. The Hgb concentration within the cytoplasm of a developing RBC provides negative feedback on both DNA and RNA synthesis. In physiologic conditions, once an optimal cytoplasmic Hgb concentration is reached (near 33–35 g/dL), negative feedback stops DNA synthesis (thus no more mitoses) and RNA synthesis (thus no more Hgb synthesis). However, until that optimal concentration is reached, the immature erythroid cells may continue to divide (and thus produce microcytes) or may make more Hgb than usual (i.e., more Hgb is needed to reach the optimal concentration in larger RBCs).[1]

d. With fresh, appropriate samples, MCV and MCHC or CHCM suggest the type of RBC that is being produced by the marrow (although in vivo postproduction processes can influence the values if severe enough).
(1) Normocytic: Erythroid cell maturation is not defective.
(2) Macrocytic: Young RBCs are present, or RBC maturation is defective.
(3) Microcytic: Extra mitoses during erythropoiesis may create smaller cells.
(4) Normochromic: Hgb synthesis is complete.
(5) Hypochromic: Hgb synthesis is incomplete (defective synthesis), or it is complete, but the cells have increased cytoplasmic fluid volume (young RBCs).
(6) Hyperchromic: RBCs were not hyperchromic when produced. Either they lost volume (in vivo or in vitro) or there is an erroneous MCHC (most likely).

3. Normocytic normochromic anemias
a. Blood film findings: RBCs are commonly uniform but may have morphologic abnormalities.
b. Most anemias begin as normocytic normochromic anemias. When marrow releases many larger or smaller RBCs with normal or decreased Hgb concentrations, then MCV or MCHC (and CHCM) change. MCV or MCHC (and CHCM) must be outside of RIs before the morphologic classification changes.
c. Persistent normocytic normochromic anemias are often nonregenerative. Many mildly or moderately regenerative anemias are normocytic normochromic because there are too few immature cells to shift the indices (mean values) outside the RIs.
d. Most anemias in horses are normocytic normochromic because their marrows release few reticulocytes. If sufficient macrocytes are released, the anemia becomes macrocytic.

4. Macrocytic hypochromic anemias
a. Blood film findings: Usually, polychromasia (except in horses), macrocytosis, and anisocytosis are present because the anemia is regenerative and due to blood loss or

hemolysis. Microscopically visual hypochromasia is not expected because reticulocytes are large and contain a normal to increased mass of Hgb (MCH is not decreased); they typically do not spread thinly enough to have increased central pallor. A macrocytic hypochromic pattern suggests the presence of immature RBCs (Figure 3.6B), but it is not specific for them (Figure 3.6E); increased polychromasia, CRP, or RC are needed for confirmation of reticulocytosis.

b. In cats, nonreticulocyte macrocytic RBCs may persist for weeks after a regenerative response to acute anemia,[9] so macrocytosis might indicate a previous regenerative response in this species. The impact on MCV would progressively decrease over time. Data were not found to confirm that these macrocytes are hypochromic as expected.

c. Dogs (e.g., schnauzers) with stomatocytosis may have macrocytic hypochromic cells.[132] Some dogs with PIMA, particularly those with collagen myelofibrosis, have macrocytic hypochromic (or normochromic) nonregenerative anemias (Figure 3.6F).[100] Hydroxyurea, a chemotherapeutic drug that impairs mitosis, has been associated with macrocytic hypochromic cells and anemia in the absence of reticulocytosis.[144]

d. The MCV and MCHC reported by automated hematologic instruments may be falsely increased and decreased, respectively. MCHC is calculated from the measured blood [Hgb] and the Hct that is calculated from a measured MCV and [RBC], so if the MCV is falsely increased (and [RBC] is accurate), the calculated MCHC will be falsely decreased.

   (1) Cell swelling during storage before testing (Figure 3.6E):[52] This occurs most frequently with mailed samples, but it occurs with any delay and can significantly increase MCV and therefore Hct by 12 h, even at 4 °C.[145] Microcytosis (e.g., in dogs with portosystemic shunts) may be masked by 24 h in samples held at room temperature.[146]

      (a) In one canine study, Hct was falsely increased by a mean of 9 percentage points (from 45 % to 54 %) after 48 h of storage at 24 °C.[145]

      (b) Under similar conditions, swelling of feline RBCs increased the mean MCV from 45 fL to 52 fL at 48 h, while the mean Hct increased from 36 % to 44 %.[142]

      (c) MCHC decreases concurrently, so hypochromasia may also be present. Hypochromic change was more prominent than macrocytic change in stored equine blood, with evidence of ghost cell formation, and storage at 4 °C produced more artifacts than storage at room temperature.[147]

   (2) In vivo hyperosmolar states (e.g., hypernatremia, hyperglycemia) can lead to increased intracellular osmolality.

      (a) When the blood within the analyzer is diluted by fluid of lower osmolality (approximately isoosmotic with plasma in health), H_2O moves into the cells and causes acute swelling and a falsely increased MCV. The MCHC (and CHCM) may become decreased.[50]

      (b) The magnitude of the difference (*dMCV*) between an analyzer MCV and MCV calculated from a spun Hct and analyzer [RBC] (Eq. 3.1b) reflects hypertonicity;[148] dMCV = measured MCV – calculated MCV. The dMCV will vary with the osmolality of buffers used in hematology analyzers.

   (3) RBC agglutination: An electronic cell counter may detect an aggregate (mostly doublets and triplets) of RBCs as one large normochromic RBC, thus falsely increasing the MCV (Figure 3.6D).

5. Macrocytic normochromic anemias
   a. Blood film findings: Polychromasia, macrocytosis, and anisocytosis are likely, but there are uncommon causes of this pattern without anisocytosis or polychromasia.
   b. Disorders or conditions
      (1) Regenerative anemias because of blood loss or hemolysis
      (2) Defective erythropoiesis; this includes primary myelodysplastic neoplasms and secondary dyshematopoiesis
         (a) FeLV-infected cats may have defective erythroid maturation that yields megaloblastic cells with defective DNA synthesis and thus decreased mitoses; megaloblastic cells mature to macrocytes.
         (b) Cobalamin or folic acid deficiencies: Cattle that graze a cobalt-deficient pasture may have a normocytic or macrocytic anemia due to cobalamin deficiency.[149] Cobalt is an essential component of cobalamin. Cobalamin deficiency depresses the activity of a methyltransferase that blocks folate metabolism by trapping a methyl group in 5-methyltetrahydrofolate. Thus, in the absence of cobalamin, a functional folate deficiency may also exist even though the serum [folate] may be WRI (5-methyltetrahydrofolate is detected in folate assays; see Folate Concentration in Dogs and Cats, Chapter 15). Cobalamin deficiency does not cause macrocytosis in dogs. Folic acid deficiency causes defective nucleic acid metabolism that could cause macrocytosis, but this is rare in spontaneous disease of domestic mammals.
         (c) Poodles with poodle marrow dyscrasia (see Abnormal Morphologic Features of WBCs, Section VII.D, in Chapter 2) have a macrocytosis and may have anemia due to another pathologic process. The pathogenesis of the macrocytosis is not established.
         (d) Erythroleukemia, myelodysplastic syndrome, or myelodysplastic/myeloproliferative neoplasms[150]
         (e) Congenital dyserythropoiesis and progressive alopecia of polled Hereford calves[68]
   c. Analytical problem: With RBC agglutination, an electronic cell counter may detect small aggregates of RBCs as single large normochromic RBCs (Figure 3.6D), thus falsely increasing the MCV and contributing to an apparent macrocytic normochromic anemia. Agglutination could be immune-mediated, induced by heparin treatment of horses, or, rarely, caused by EDTA in vitro. Particles (agglutinates) larger than an upper threshold for RBC volume are excluded.
6. Microcytic hypochromic anemias (Figure 3.6C)
   a. Blood film findings: With Fe deficiency, a common cause, expect hypochromasia, microcytosis (difficult to judge), leptocytosis including folded RBCs, codocytosis, and anisocytosis. Other poikilocytes may include ovalocytes, schistocytes, and keratocytes. Polychromasia may be present, but it is typically less than expected for the degree of anemia.
   b. Microcytosis and hypochromasia may be due to defective Hgb synthesis caused by the following:
      (1) Fe deficiency (see Blood Loss Anemias, Section II.B)
      (2) Copper deficiency in dogs;[151] however, an experimental Cu deficiency in dogs produced a normocytic normochromic anemia[152]
      (3) Potentially, vitamin B_6 (pyridoxine) deficiency

c. Chronic hepatopathies (hepatic failure, chronic hepatitis, possibly hepatocellular carcinoma) or portosystemic shunting may cause microcytic hypochromic anemias, typically without prominent visual hypochromasia, but a microcytic normochromic pattern is more likely.
d. With chronic inflammation, the typical normocytic normochromic AID may become microcytic and hypochromic.[153]
e. Congenital dyserythropoiesis and myopathy syndrome in Labrador retrievers and erythropoietic porphyria in cats and cattle are rare causes.[67,83]
f. Some dogs with breed-related microcytosis also have decreased MCHC (see next section) whether anemic or not.
g. The MCV may be decreased by certain sample or patient conditions (Table 3.3 and the following Sections 7 and 10).

7. Microcytic normochromic anemias
   a. Blood film findings vary from those seen in microcytic hypochromic anemia to those seen in normocytic normochromic anemia.
   b. Causes of microcytosis
      (1) Fe deficiency: Prior to causing a microcytic hypochromic anemia in dogs, Fe deficiency may produce a microcytic normochromic anemia with a decreased MCH (not MCHC). Microcytosis without hypochromasia is expected in cats[154] and horses with Fe-deficiency anemias, in contrast to dogs, pigs, ruminants, and camelids.
      (2) Chronic hepatopathies (hepatic failure, chronic hepatitis, hepatocellular carcinoma) or portosystemic shunts: The cause of microcytosis is not known, and it often occurs without anemia, but data suggest a defect in Fe transport to RBC precursors. MCH is decreased, and MCHC (CHCM) is often mildly decreased in dogs, with little or no microscopic hypochromasia unless there is a concurrent decrease in Fe stores, e.g., in young dogs with a shunt.
      (3) Dyserythropoietic anemia and myopathy syndrome in English springer spaniels or Labrador retrievers (rare)[66,67,82]
      (4) With chronic inflammation, the typical normocytic normochromic AID may become microcytic and normochromic.[153]
      (5) Microcytosis with or without anemia has been reported in cats with feline infectious peritonitis (FIP).[81]
      (6) Some healthy Akitas, shibas, Jindos, chow-chows, and shar-peis have lower MCV values than do dogs of other breeds, and some may also have mild decreases in MCHC.[84,155] Akitas with low-K^+ RBCs have lower MCV than Akitas with high-K^+ RBCs.[155]
      (7) Foals and kittens may have lower MCV values than do adult animals of the respective species;[85,87] this likely relates to limited Fe stores during growth.
8. Normocytic hypochromic anemias: If found, one must consider that the data may be inaccurate or that the RIs may be inappropriate. RBCs may be hypochromic because of immaturity, Fe deficiency, or cell swelling, but the MCV may not have changed enough to be outside the RI.
9. Macrocytic or normocytic hyperchromic anemias: In theory, it is not physiologically possible to produce hyperchromic RBCs because Hgb synthesis stops in a RBC precursor when an optimal [Hgb] is reached within its cytoplasm. The MCHC is expected to be falsely increased (see the following Section 12). Compare the MCHC to the CHCM, if available. Consider causes of macrocytosis when present.

10. Microcytic hyperchromic anemias
    a. Blood with marked eccentrocytosis and pyknocytosis may have an increased MCHC because oxidative condensation of Hgb and fusion of cell membranes cause a loss of cell volume without a proportionate loss of cell Hgb.[101,156] These cells may also be microcytic. However, they are usually not numerous enough to increase the CHCM and MCHC.
    b. If the MCHC (or CHCM) is falsely increased for reasons that do not cause microcytosis, potential causes of pathologic microcytosis should be considered.
    c. Note: Spherocyte populations in domestic species appear hyperchromic and small, microscopically, because of their thickness, but MCHC, CHCM, and MCV are WRI.
    d. Falsely low MCV and high MCHC (CHCM) may be produced when RBCs are in hypoosmolal plasma.[50,157] RBCs adjust in vivo to the hypoosmolal environment caused by hyponatremia and hypochloremia by having decreased cytoplasmic osmolality. When mixed with a diluent prior to counting, osmosis results in H_2O leaving the RBCs and thus decreasing volume of RBCs.
11. Increased MCHC or CHCM with or without anemia or microcytosis
    a. Increased MCHC is almost always artifactual, and MCH may also be falsely increased. CHCM is more reliable but can be falsely increased. Causes of falsely increased MCHC, CHCM, or MCH:
        (1) Pathologic hemoglobinemia: With in vivo hemolysis, the measured [Hgb] would include plasma Hgb, which would be attributed to intact sample RBCs, thus falsely increasing MCHC and MCH. CHCM would not be increased because it is assessed directly by light scatter. A more accurate MCHC could be calculated from a [Hgb] adjusted for Hgb_{delta}.
        (2) Oxyglobin: Like pathologic hemoglobinuria, free Hgb from use of Hgb-based O_2 carriers causes an overestimation of intracellular Hgb and falsely increases the MCHC and MCH. The CHCM is not affected. A more accurate MCHC could be calculated from a [Hgb] adjusted for Hgb_{delta}.
        (3) In vitro hemolysis: With in vitro lysis, the sample Hct and [RBC] are falsely decreased but the blood [Hgb] is accurate, thus the MCHC and MCH are falsely increased (Eq. 3.1b). In vitro hemolysis occurs for many reasons, including traumatic collection, forceful transfer from syringe to blood tube, excessive mixing or shaking of the sample, jarring transport, exposure to high or freezing temperatures, and delayed testing; concurrent lipemia or increased RBC fragility promotes in vitro hemolysis.
        (4) Spectral interferences in the blood Hgb assay: Interferences that can produce a falsely increased blood [Hgb] and therefore MCHC and MCH include lipid droplets in grossly lipemic samples, pigments in markedly icteric samples, nuclei or intact WBCs in samples with extreme leukocytosis, Heinz bodies (causing incomplete RBC lysis), many RBCs containing Hgb crystals (rare), and precipitates of immunoglobulins (e.g., IgA or IgM paraproteins) that may occur with certain buffers and methods.[158,159] Of these, many Heinz bodies or RBC Hgb crystals can also falsely increase the CHCM, although CHCM is often not increased by Heinz bodies; if it is, the increase is mild compared to the false increase in MCHC, thus yielding an increased Hgb_{delta}.[45,160,161]

(5) As discussed in the preceding Section 10, the MCHC and CHCM may be falsely increased because of cell shrinkage related to in vivo hyponatremia followed by cell contact with a relatively hyperosmolal diluent in the analyzer.

C. Pathophysiologic classification

1. This is based on the pathologic mechanism or process that produced the anemia. Multiple pathologic processes may contribute to an anemia.
   a. Blood loss anemias: These can be acute (hours to days), chronic (weeks to months), or acute on chronic.
      (1) In *external blood loss anemias*, RBCs are lost from the body, including into the alimentary or urinary tract (which may seem internal, but RBC content is lost from the body). Chronic external blood loss can result in Fe deficiency.
      (2) In *internal blood loss anemias*, RBCs move from the intravascular to the extravascular space, commonly into subcutaneous tissue or peritoneal or pleural cavities from which Fe can typically be recycled.
   b. Hemolytic anemias
      (1) With *extravascular hemolysis*, tissue macrophages engulf and destroy RBCs within the normal circulatory path of blood traveling through the spleen, liver, or bone marrow. This is different from hemorrhage, when RBCs leave the circulation before phagocytosis. (Rarely, a RBC is seen within a monocyte or macrophage in a blood smear.) Extravascular hemolysis occurs without detectable Hgb release to plasma.
      (2) With *intravascular hemolysis*, there is RBC lysis and Hgb release directly into the plasma without macrophage involvement. Extravascular hemolysis then mediates clearance of RBC remnants produced by intravascular hemolysis.
   c. Anemias caused by decreased RBC production
      (1) Inflammatory diseases
      (2) Chronic renal disease
      (3) Marrow hypoplasia or aplasia
      (4) Erythroid hypoplasia or ineffective erythropoiesis
2. The pathophysiologic classification system is frequently used in one of these two ways:
   a. It serves as a broad differential diagnosis list for further refinement or to answer questions such as "What are the basic causes of anemias?" (Figure 3.17).
   b. It is used to group specific diseases based on the method or methods by which they cause anemia.

NONREGENERATIVE ANEMIAS

I. General concepts

A. The major reason for a persistent nonregenerative anemia is decreased RBC production; defective erythropoiesis can also contribute. Since RBC life spans of domestic mammals are generally 2–5 mo, an anemia takes several weeks to months to develop if it is caused only by decreased erythropoiesis.

1. For example, the RBC life span in dogs, including greyhounds, is about 100 d.[162] In health, about half of a dog's RBCs are > 50 d old and about half are < 50 d old. If a disease stopped erythropoiesis completely and did not alter RBC life span, it would take 25 d for the dog's Hct to drop from 40 % to 30 %, and about 50 d to drop from 40 % to 20 %.

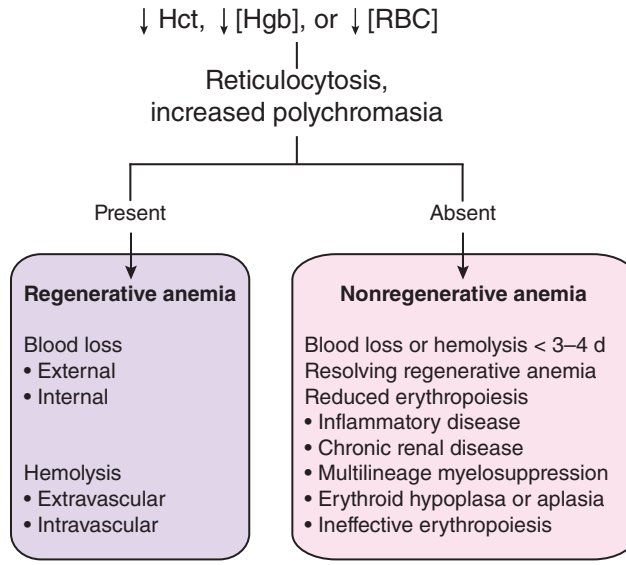


Figure 3.17. An approach to problem-solving anemias. After anemia has been detected or confirmed, the presence or absence of a regenerative response is determined from a RC, CRP, or polychromasia (see Anemia, Section II.A).

- If the anemia is regenerative (increased RC, CRP, polychromasia), then the anemia is probably due to blood loss or hemolysis. History, physical exam, imaging, and several laboratory tests are useful for differentiating these two general mechanisms. Protein assessment may provide evidence of blood loss (nonselective hypoproteinemia), whereas blood smear evaluation, clinical chemistry results, or urinalysis results may provide evidence of intravascular or extravascular hemolysis, e.g., RBC organisms, hemolysis-associated poikilocytes, ghost cells, hyperbilirubinemia with a predominance of Bu, or bilirubinuria.
- If moderate to marked anemia of several days' duration is nonregenerative or inappropriately (mildly) regenerative, it supports reduced erythropoiesis. A mild anemia without reticulocytosis could also be a resolving regenerative anemia with increased erythropoiesis and most reticulocytes maturing in the bone marrow before release. History, physical exam, imaging, CBC, clinical chemistry results, and urinalysis are used to identify inflammatory disease or chronic renal disease. If the anemia cannot be attributed to inflammatory disease or chronic renal failure, bone marrow examination is often indicated to determine if there is nonselective marrow suppression, selective erythroid hypoplasia or aplasia, or ineffective erythropoiesis because of precursor destruction or abnormal maturation.

2. Because cat RBCs have shorter life spans (approximately 70 d), such anemias develop more quickly.
3. Production-failure anemia develops more slowly in horses and cattle because their RBCs have longer life spans (approximately 150 d).

B. Most diseases do not stop RBC production completely but only decrease the rate of production. Therefore, nonregenerative anemias may take even longer to develop. However, many diseases that reduce erythropoiesis also shorten RBC life span, and thus anemia may develop more quickly than expected from reduced erythropoiesis alone.

C. Most animals with nonregenerative anemias have been anemic for several weeks before clinical signs are detected, so the anemia indicates a chronic disorder. Severity of a nonregenerative anemia depends on duration of the disease, degree of decreased erythropoiesis, and presence of other processes that shorten RBC life span.

D. When a persistent nonregenerative anemia is detected, most RBCs in the animal's blood were produced when the animal had the disease that caused the anemia. Thus, characteristics of the circulating RBCs may help determine the cause of the persistent nonregenerative anemia (see Anemia, Section II.B).

II. Disorders that cause nonregenerative anemias (Table 3.10)

A. Disorders causing selective erythroid hypoplasia or ineffective erythropoiesis (without generalized marrow hypoplasia) (see Chapter 6 and Tables 6.3 and 6.4)

1. Inflammatory disease

a. Inflammation causes AID, which is also called anemia of chronic inflammation or anemia of chronic disease. However, the pathogenesis of anemia in chronic disease is not always related primarily to inflammation, so "AID" is a more specific designation, reflecting a particular constellation of mechanisms. AID is a common cause of nonregenerative anemia in domestic mammals and varies from mild to moderate severity. Typically, it is a normocytic normochromic anemia, but it can be microcytic.

b. AID is of relatively little clinical significance after it is recognized. Most diagnostic efforts are directed toward the primary disease, not toward the anemia which will resolve with resolution of the underlying disorder.

c. Almost any chronic disorder with an inflammatory component, infectious or noninfectious, initiates the processes that cause anemia. AID develops faster in species with shorter RBC life spans (e.g., cats).

d. The pathogenesis of AID involves these three concurrent mechanisms initiated by inflammation:[163]

Table 3.10. Disorders and conditions that cause nonregenerative anemias

Disorders causing selective erythroid hypoplasia or ineffective erythropoiesis
Inflammatory disease (primarily chronic)
Infectious:* bacterial, fungal, viral, protozoal, parasitic
Noninfectious:* immune, toxic, neoplastic (usually malignant ± necrosis)
Renal disease (chronic)*
Endocrinopathies:* hypothyroidism, hypoadrenocorticism, hypoandrogenism, hypopituitarism
Nutrient deficiencies: Fe,* copper, cobalt, folate, vitamin B_{12} (cobalamin), starvation
Chronic hepatic disease or insufficiency (including portosystemic shunts)*
Pure red cell aplasia
FeLV-induced erythroid hypoplasia[a]
PIMA*
Congenital dyserythropoietic anemias of English springer spaniels, polled Hereford calves, or Murray Grey calves
Disorders causing persistent generalized marrow hypoplasia or aplasia (Table 6.4)

* A relatively common disorder, condition, or mechanism

[a] FeLV may cause hypoplasia or ineffective erythropoiesis.

Note: Blood loss or hemolysis of less than 3–4 d duration and resolving regenerative anemias that have become mild may mimic nonregenerative anemias from other causes.

(1) Shortened RBC survival
  (a) Pathologic events are not understood entirely but are associated with increased [IL-1].
  (b) Oxidant and enzymatic damage to RBC membranes and subsequent binding of immunoglobulin molecules may accelerate the removal of RBCs.[164]
(2) Impaired Fe mobilization or utilization
  (a) Hepcidin production by hepatocytes is stimulated by IL-6, and the binding of hepcidin to ferroportin in cell membranes internalizes ferroportin. Without membrane ferroportin, enterocytes cannot transport dietary Fe into the blood. More importantly with regard to the daily mass of available Fe, macrophages cannot export Fe, so less of the Fe from destruction of senescent RBCs is released from splenic and other macrophages for use.[165] This impairs Hgb synthesis and erythropoiesis. This can become severe enough for erythropoiesis to yield microcytic cells.
  (b) Alterations in ferritin production and alterations in transferrin receptors also increase Fe storage and therefore decrease the availability of Fe for Hgb synthesis.
(3) Impaired RBC production
  (a) Erythroid cells become refractory (less responsive) to increased Epo because of the effects of inflammatory cytokines (IL-1, interferon, and TNF) on precursors. These effects might include downregulated expression of Epo receptors, promotion of apoptosis, or decreased production of other hematopoietic factors.
  (b) The Epo response to anemia is blunted; hypoxia-induced Epo production is increased but not as much as expected. This is due to actions of IL-1, TNF, and transforming growth factor β.

e. Bone marrow evaluation is rarely indicated. These laboratory findings support the conclusion that an animal has AID:
(1) Mild to moderate normocytic normochromic anemia, often with little to no poikilocytosis; decreased CHr and MCVr may precede anemia and persist until effective therapy resolves inflammatory suppression
(2) Chronic inflammatory leukogram: mature neutrophilia, lymphocytosis, or monocytosis, with or without eosinophilia
(3) Hyperproteinemia due to increased concentrations of γ-globulins or positive acute-phase proteins
(4) Hypoferremia, serum [ferritin] WRI to increased, and adequate to increased hemosiderin in marrow (except for cats), spleen, or liver
(5) Marrow with mild to moderate erythroid hypoplasia, mild to marked granulocytic hyperplasia, and possibly plasmacytosis in addition to prominent hemosiderin in species other than cats

2. Renal disease (chronic)
a. Most patients with chronic renal disease are anemic. The anemia is typically normocytic normochromic and slight to moderate in severity.
b. Pathogenesis of anemia
(1) Inadequate Epo production is a major cause (Epo therapy is effective at increasing Hct). Chronic renal disease damages the kidneys enough to limit Epo production, so [Epo] does not increase appropriately (though it may not be

decreased). Thus, stimulation of RBC production is inadequate for the degree of anemia.

(2) Impaired renal clearance of hepcidin and potentially concurrent inflammatory disease may lead to increased [hepcidin], decreased Fe availability, and decreased erythropoiesis as described for inflammation. Loss of transferrin and Fe with proteinuria might also impair erythropoiesis.

(3) Substances not cleared by the kidneys may alter RBCs and decrease their life span by mechanisms that include eryptosis (the RBC equivalent of nucleated cell apoptosis).

(4) Decreased marrow response to Epo has been described.

(5) Other factors: hemorrhage caused by uremic ulcers or vascular damage; poor nutritional status

c. Laboratory findings typically include evidence of chronic renal disease or dysfunction, such as azotemia, inappropriately low urine specific gravity (e.g., isosthenuric range), and electrolyte disturbances. Bone marrow evaluation is rarely indicated.

3. Endocrinopathies

a. Hypothyroidism, seen primarily in dogs, may cause a mild normocytic normochromic anemia.

(1) Pathogenesis of the anemia: Decreased [total thyroxine] and [total triiodothyronine] result in a decreased metabolic rate and thus a decreased need for O_2 in tissues. The decreased need for O_2 leads to decreased Epo production and thus less RBC production. A new homeostasis develops in which metabolic needs for O_2 are met by a lower blood [RBC].

(2) Laboratory findings, besides the mild nonregenerative normocytic normochromic anemia, include evidence of thyroid dysfunction such as decreased [total thyroxine], decreased [free thyroxine], and increased [thyroid-stimulating hormone]. Bone marrow evaluation is rarely indicated.

b. Hypoadrenocorticism, seen primarily in dogs, may cause a mild to sometimes marked anemia.

(1) Pathogenesis of the anemia is not established, but glucocorticoids have been reported to stimulate erythropoiesis in vitro, so their absence may be relatively marrow suppressive. Gastrointestinal blood loss contributes to anemia in some dogs and may be associated with melena or hematemesis when severe.[166,167] With acute gastrointestinal hemorrhage, the anemia is expected to become regenerative with successful therapy.

(2) Laboratory testing most commonly reveals a mild nonregenerative normocytic normochromic anemia, although it may be masked by hypovolemia-induced hemoconcentration. Other findings include evidence of adrenal dysfunction such as hyponatremia, hyperkalemia, azotemia, hypocortisolemia, and lack of a steroid leukogram (no lymphopenia or suppressed [eosinophil] and sometimes increases in either or both). Bone marrow evaluation is rarely indicated.

c. Other hormone deficiencies that may cause anemia by decreased erythropoiesis are hypoandrogenism and hypopituitarism.

4. Nutrient deficiencies: Deficiencies of nutrients required for erythropoiesis can cause ineffective erythroid hyperplasia and dyserythropoiesis.

a. Fe deficiency occurs because of chronic external blood loss (e.g., alimentary tract blood loss because of ulcers or parasites, cutaneous blood loss because of fleas or ticks,

excessive blood donation) or inadequate dietary Fe intake (typically in neonates). The cause has not always been clear in llamas.[168] When diagnosed, the anemia is classically microcytic hypochromic or microcytic normochromic (for its pathogenesis, see Blood Loss Anemias, Section II.B). Bone marrow evaluation is rarely indicated.

b. Copper deficiency is uncommon in domestic mammals but has been reported in pigs, dogs, and potentially llamas.[169] RBC abnormalities develop because of defective Fe transport, but increased oxidative injury and hemolysis may contribute to anemia in sheep.[170]
   (1) Ceruloplasmin (ferroxidase) and hephaestin are related Fe oxidases that contain copper and promote the conversion of Fe^{2+} to Fe^{3+} during Fe^{2+} transport out of macrophages and enterocytes, respectively. If an animal is deficient in copper, there is less ferric oxidase activity, less Fe^{2+} absorption from the intestine, less release from macrophages, less Fe^{2+} available for heme synthesis, and thus defective Hgb synthesis. Because of the copper deficiency, there is a functional Fe deficiency, and thus a microcytic hypochromic anemia might develop. Serum Fe concentrations should not be decreased.
   (2) In an experimental study, copper-deficient dogs developed normocytic normochromic anemias.[152] The pathogenesis of anemia was not established.
   (3) An iatrogenic copper deficiency was created when trientine hydrochloride was used to treat copper storage disease in a Bedlington terrier.[151] A microcytic hypochromic anemia persisted after treatment for Fe deficiency. The anemia resolved and the RBC indices improved when the therapy for copper storage disease ceased. Interpretation of the case data was complicated by evidence of hepatic dysfunction.
   (4) Microcytic hypochromic anemias do develop in copper-deficient pigs. Copper deficiency was considered a likely contributor to hypochromic anemia in two llamas, but this was unproven, and methods used to generate the partial erythrogram data were not provided.[169]

c. Deficiencies of folate or cobalamin (vitamin B_{12}) can cause abnormal RBC development and anemia because they are required for DNA synthesis, but such disorders are rarely found in domestic mammals. Folate and cobalamin deficiencies cause macrocytic anemia in people, but no relationship between these deficiencies and anemia or macrocytosis was found for dogs.[171] Severe cobalamin deficiency associated with congenital cubam defects in dogs (below) has repeatedly been associated with normocytic (not macrocytic) anemia. A cat with a congenital cobalamin deficiency had normocytic RBCs.[172]
   (1) Selective cobalamin malabsorption with proteinuria (Imerslund-Gräsbeck syndrome): Mutations in the genes for cubilin (*CUBN*) and amnionless (*AMN*), the proteins forming the cubam receptor responsible for ileal absorption of cobalamin and renal tubular resorption of several proteins, have been defined in giant schnauzers (*AMN*), Australian shepherds (*AMN*), border collies (*CUBN*), beagles (*CUBN*), and Komondors (*CUBN*).[173] A beagle cross with typical findings of a cobalamin receptor defect was heterozygous for the border collie mutation and the beagle mutation.[174] Reported hematologic changes in these dogs include ineffective hematopoiesis (erythroid and myeloid hyperplasia) and dyshematopoiesis with normocytic normochromic nonregenerative anemia that may be accompanied by rubricytosis, mild erythroid dysplasia in blood and

marrow, neutropenia, hypersegmented neutrophils, and large band metamyelocyte neutrophils in the bone marrow.[175,176] Other laboratory findings in these young dogs with failure to thrive and severe hypocobalaminemia may include mild tubular proteinuria of cubam ligands and metabolic changes caused by impaired function of cobalamin-dependent enzymes: methylmalonic aciduria/acidemia, hyperammonemia, ketoacidosis, ketonuria, and hyperhomocysteinemia.

(2) Cattle that develop cobalamin deficiency from grazing on cobalt-deficient soil may develop normocytic normochromic anemia.[177]

(3) Cats with experimental folate deficiency had megaloblastic marrow erythroid cells but neither macrocytosis nor anemia.[178] However, two folate-deficient cats on a folate-deficient vegan diet had macrocytic anemias that reportedly resolved (anemia and macrocytosis) after folic acid supplementation for 30 d.[179]

d. Pyridoxine (vitamin B_6) deficiency: A dietary pyridoxine deficiency in growing kittens resulted in anemia, but the features and pathogenesis of the anemia were not described.[180]

5. Chronic hepatic disease or insufficiency (including portosystemic shunts) are frequently associated with mild to moderate anemia in domestic mammals. Typically, the anemias are normocytic normochromic, but decreased MCHC values have been reported, and some dogs have a microcytic normochromic or microcytic hypochromic anemia.
   a. Pathogenesis of the anemia: The anemia could be an AID in some cases. Other potential mechanisms include defective amino acid and protein synthesis and abnormal lipid metabolism affecting RBC lipid content and life span. In dogs with hepatic insufficiency, the microcytosis is not caused by total body Fe deficiency; however, defective protein synthesis may create a functional Fe deficiency because of defective Fe transport.
   b. Besides the anemia, laboratory findings may include enzymatic evidence of hepatic disease or evidence of hepatic dysfunction: decreased serum [urea], hypoproteinemia, hypoalbuminemia, increased serum [bile acids], hyperammonemia, or ammonium biurate crystalluria. Bone marrow evaluation is rarely indicated.
6. Pure red cell aplasia is a descriptive term for disorders in which a nonregenerative anemia is caused by marked erythroid hypoplasia or aplasia, but other hematopoietic cell lines are not diminished. It may be primary (presumed immune mediated) or secondary to other disorders, including FeLV infection, and has been recognized in dogs, cats, and people. Bone marrow evaluation is necessary for a diagnosis. Refer to Bone Marrow Classifications, Section II.B, in Chapter 6.
7. FeLV may infect hematopoietic cells or marrow stromal cells and cause erythroid hypoplasia or aplasia (previous entry), but it can also transform a cell into a neoplastic cell line.
   a. If erythroid or multilineage progenitor cells undergo neoplastic transformation, erythroid (and other) cells may not mature, or they may die or be destroyed before maturing to RBCs, and thus anemia develops because of decreased effective erythropoiesis. The neoplasm may cause hypoplasia of nonneoplastic cells.
   b. Laboratory findings in FeLV-infected cats may include a mild to severe, nonregenerative anemia that is normocytic normochromic or macrocytic normochromic. Inappropriate rubricytosis may be present, especially in myelodysplastic syndrome with erythroid predominance (MDS-Er). Marrow

findings vary from erythroid hypoplasia or aplasia to a neoplasm of any marrow cell lineage. Megaloblastic erythroid cells may be found in blood or marrow (megaloblastic anemia). Megaloblastic cells have asynchronous maturation of nuclei and cytoplasm, with cytoplasm appearing mature compared to nuclei. The defective maturation produces larger erythroid precursors with atypically large nuclei for the degree of cytoplasmic maturation. Nuclei commonly have exaggerated euchromatin regions that persist to late rubricyte stages, and cells with relatively large nuclei may have mature cytoplasm with prominent Hgb. Megaloblastic precursors may become macrocytes. For information about FeLV detection, see Bone Marrow Classifications, Section II.B.1.b.(3), in Chapter 6.

8. PIMA in dogs and cats[181,182] appears to be caused by an immune-mediated attack on erythroid precursors at more-mature stages than the cells suppressed in pure red cell aplasia (see Bone Marrow Classifications, Sections I.A.2 and II.B.1, in Chapter 6). Instead of erythroid aplasia, there is a bone marrow response to Epo with expansion of erythroid precursors up until the maturation stage at which the precursors are destroyed, i.e., there is ineffective erythropoiesis.
   a. PIMA can occur with IMHA, in which case IMHA is nonregenerative but immune-mediated destruction is apparent from laboratory findings without assessing the bone marrow. PIMA also occurs with no evidence of hemolysis (i.e., "pure PIMA"), which generally requires bone marrow evaluation to differentiate it from other causes of moderate to marked nonregenerative anemia.
   b. Laboratory findings typically show a normocytic normochromic anemia, but a subset of affected dogs have macrocytic hypochromic or normochromic anemia with ovalocytosis, usually when collagen myelofibrosis is present. When associated with IMHA, there may be a spherocytosis and a positive DAT, but these are absent in "pure PIMA" cases. Bone marrow findings are described in Chapter 6.
9. Dyserythropoietic anemia and myopathy syndrome in English springer spaniels[66,82] is characterized by dyserythropoiesis and polymyopathy with weakness, exercise intolerance, megaesophagus associated with regurgitation, and varying degrees of cardiomegaly. Clinical findings include regurgitation and decreased muscle mass in young dogs (3 mo to 4 yr at initial evaluation).
   a. A likely causative *EHBP1L1* frameshift mutation with autosomal recessive inheritance has been identified, but the pathogenesis of the disorder is unknown.[82]
   b. Laboratory findings include microcytic normochromic nonregenerative anemia (also described as hypochromic, but MCHC was WRI in that report) with inappropriate rubricytosis (many small metarubricytes), codocytosis, and a variety of other described poikilocytes and inclusions. Marrow findings include erythroid hyperplasia and macrophages containing abundant hemosiderin.
10. Dyserythropoietic anemia and myopathy syndrome in two 5-yr-old male Labrador retriever siblings was similar to that in English springer spaniels, but without cardiomyopathy.[67] Affected dogs had microcytic mild anemias with inappropriate rubricytosis (no reticulocytosis), poikilocytosis including codocytes, and right-shifted ineffective erythropoiesis associated with phagocytosis of metarubricytes. A nonsense mutation in *EHBP1L1* was identified in both retrievers, but it was absent from two of the English springer spaniels previously described with dyserythropoietic anemia and myopathy (different defects in the same gene).[183]
11. Congenital dyserythropoiesis and progressive alopecia of polled Hereford calves[68] is a syndrome with macrocytic normochromic anemia and cutaneous lesions characterized

by a hyperkeratotic dermatitis with dyskeratosis in stratum spinosum and follicular infundibula.

a. The pathogenesis of the disorder is unknown but appears to be related to impaired cytokinesis during terminal cells divisions.
b. Laboratory findings: Besides the macrocytic (MCV near 54 fL in affected calves and MCV near 41 in unaffected calves) normochromic anemia (Hct near 16 %), there was rubricytosis and mild reticulocytosis that appeared inappropriate for the severity of anemia. Hyperferremia was present with normal TIBC. Marrow findings included erythroid hyperplasia, multinucleated erythroid cells, irregular chromatin patterns, and slight megalocytosis. There appeared to be a maturation arrest in the late rubricyte stage, but with cytoplasmic staining indicating nearly complete Hgb synthesis in the rubricytes. Nuclear buds were common in rubricyte nuclei. Hemosiderin was abundant.

12. Congenital dyserythropoietic anemia of Murray Grey calves has been minimally described,[69] but affected calves have retarded growth, exercise intolerance, weakness, and icterus by 1–8 wk of age, and they may be found dead. Laboratory findings include severe anemia with rubricytosis but apparently without reticulocytosis. Binucleate nRBCs were common in blood, and bone marrow was characterized by marked erythroid hyperplasia (ineffective) and many large multinucleate erythroid precursors. Others have reported multinucleate hepatocytes. Additional findings supported hemolysis. The defect in these calves has not been identified, but the disorder resembles congenital dyserythropoietic anemia in people.[184]

B. Disorders causing persistent generalized marrow hypoplasia or aplasia (see Table 6.4 and Chapter 6, Section II.A, Bone Marrow Classifications): These disorders decrease production of erythroid and other cell lineages, thus causing multiple cytopenias from damage to hematopoietic cells or the marrow's microenvironment. Anemia may develop later than other cytopenias because circulating RBCs have a much longer life span (months) than platelets (~1 wk) and neutrophils (~1 d). The following disorders may cause marked anemia if they persist long enough for many RBCs to reach senescence and be cleared from circulation without adequate replacement. Concurrent mechanisms that shorten RBC life span may accelerate the development of anemia.

1. Aplastic anemia (markedly hypoplastic or aplastic pancytopenia) is idiopathic in domestic mammals, but might be caused by toxicants, *Ehrlichia canis* infection in dogs, or immune-mediated myelosuppression. Immunosuppressive therapy may be instituted if no cause is found.
2. Infectious agents may suppress hematopoiesis through direct hemic cell infection, myelitis, or secondary effects such as cytokine-induced marrow suppression. Suppression may occur with chronic ehrlichiosis, disseminated mycoses (e.g., histoplasmosis), viral infections (e.g., EIAV), or protozoal infections (e.g., leishmaniasis in dogs or rarely cats, or cytauxzoonosis in cats). Infections with *Cytauxzoon felis* are somewhat unique among these infectious agents because the agent has piroplasms in RBCs and schizonts in macrophages.[185] Clinically, cytauxzoonosis caused by *C. felis* is often a rapid and highly fatal disease, but some cats survive natural infections.[186,187]
   a. When blood RBCs contain the piroplasms, cats may or may not be anemic, and they may be clinically recovered but with chronic parasitemia. When present, anemias are often nonregenerative and mild to marked, without marrow erythroid hyperplasia; these anemias are probably caused by AID and damage to marrow, spleen, and liver. Some cats may be in a preregenerative state.

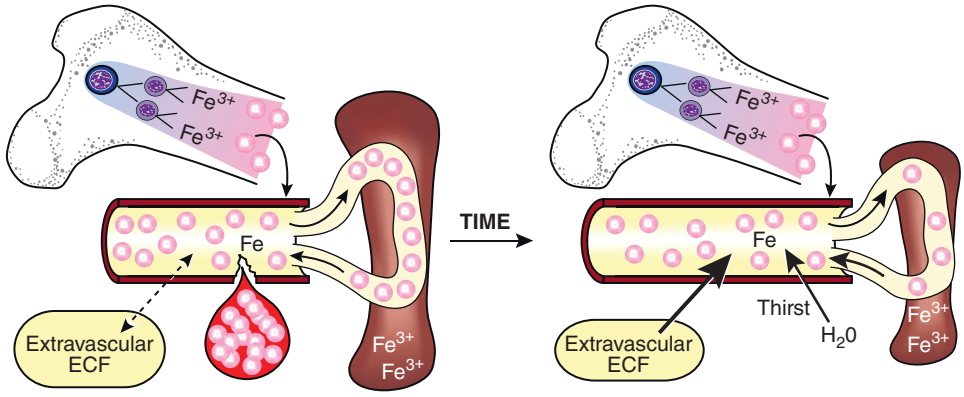
b. Fever and icterus are common clinical findings. The moderate to marked hyperbilirubinemia and resultant bilirubinuria may be caused primarily by hepatic damage and cholestasis, but erythrophages are found in many organs, supporting at least a minor contribution of hemolysis.[185,188]
c. Other laboratory findings may include the following: thrombocytopenia, leukopenia, low-normal or decreased serum alkaline phosphatase activity,[188] and large macrophages laden with schizonts in blood films or in cytologic samples from spleen, lymph nodes, bone marrow, or liver.
d. Some cats infected with *Cytauxzoon* spp. that are genetically distinct from *C. felis* have had regenerative anemias.[189] Some *Cytauxzoon* spp. may induce mild disease.

3. See Section II.A of Bone Marrow Classifications in Chapter 6 for information about toxicants/drugs and other causes of marrow suppression that may cause a nonregenerative anemia with or without other cytopenias.

BLOOD LOSS ANEMIAS

I. Causes of blood loss
   A. Hemorrhage
      1. Blood vessels damaged by trauma, ulceration, a neoplasm, or other means
      2. Acquired or congenital coagulation factor deficiencies or von Willebrand disease
      3. Thrombocytopenia (marked) or severely impaired platelet function
   B. Parasitism: hookworms and whipworms (dogs), haemonchosis and ostertagiasis (ruminants), coccidiosis, ticks, bloodsucking lice, and fleas (dogs, cats, and calves)
   C. Removal of blood that is to be used for a transfusion

II. Classifications based on duration and location
   A. Acute blood loss anemia
      1. This occurs when blood is lost from vessels over minutes to days, but the result of acute blood loss may be apparent for at least two weeks. Anemia results from the dilution of RBCs that remain in vessels (Figure 3.18).
         a. The severity of an anemia caused by hemothorax or hemoperitoneum may be diminished by resorption (autotransfusion) of about 65 % of RBCs within 2 d and 80 % within 1–2 wk.[190] Also, animals do not become Fe depleted, because RBCs are absorbed (autotransfusion) or destroyed and the Fe is reutilized.
         b. Sudden anemia creates tissue hypoxia that stimulates Epo production. If marrow is responsive, reticulocytosis should be present 3–4 d after blood loss (except in horses).
      2. Clinical data that support a conclusion that an anemia is caused by acute blood loss include the following major diagnostic features:
         a. Blood loss was observed (historical or physical examination).
            (1) Gross external hemorrhage is seen. If there is gastrointestinal hemorrhage, then the feces may be tarry (melena) or occult-blood positive (heme present). If there is urinary tract hemorrhage, there are RBCs in the urine sediment or a heme-positive reaction.
            (2) Hemothorax or hemoperitoneum is documented.
         b. Regenerative anemia if sufficient time for response
         c. Hypoproteinemia with a proportionate decrease in [albumin] and [globulin]: Hypoproteinemia caused by intracavitary hemorrhage may not be as severe as external hemorrhage because proteins can return to plasma via lymphatic vessels.



A. Sudden loss of blood from a vessel creates hypovolemia but Hct % and protein concentration of remaining blood does not change.

B. Shift of ECF into vessels dilutes RBCs, causing anemia and hypoproteinemia; splenic contraction reduces severity of the anemia.

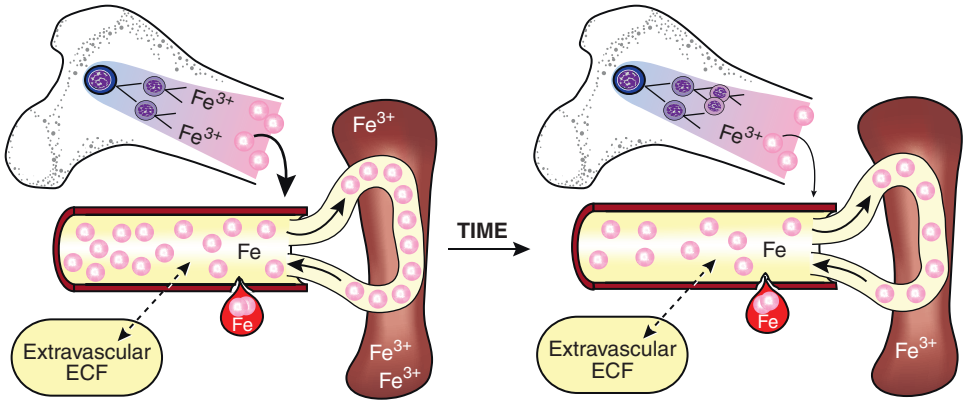
Figure 3.18. RBC kinetics of acute blood loss.

A. Immediately after whole blood is lost, Hct and [total protein] should not change because RBCs, proteins, and plasma are lost proportionately, but blood volume is decreased.

B. Hypovolemia stimulates thirst to replenish ECF volume and induces movement of ECF from the extravascular space to the intravascular space, thus expanding blood volume and diluting RBCs (and plasma proteins) so that anemia (and hypoproteinemia) develops. The degree of anemia depends on the quantity and duration of hemorrhage and the time from the onset of hemorrhage. Splenic contraction diminishes the severity of the anemia because splenic blood is rich in RBCs (especially in horses and dogs).

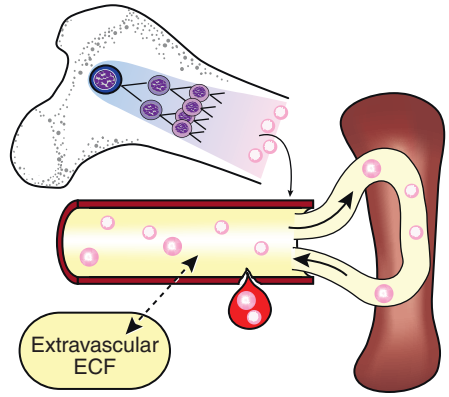
B. Chronic blood loss anemia that leads to Fe-deficiency anemia

1. Chronic blood loss occurs when blood is lost from the body (including into the gastrointestinal tract or urinary tract) over several weeks to months. Anemia results from a combination of factors but is primarily the result of Fe deficiency (Figure 3.19).
   a. Once Fe deficiency is present, maturation and release of RBCs are impaired such that those being lost cannot be replaced rapidly enough despite erythroid hyperplasia. Ongoing blood loss in the presence of Fe deficiency also contributes to anemia, but the amount of blood loss itself typically is not the major factor. If Fe were available, regeneration would correct or ameliorate the anemia.
   b. During Fe deficiency, RBCs become more fragile and less deformable;[191] these changes decrease RBC life span. The reduced deformability has been attributed to increased membrane rigidity, but the reason for the rigidity was not explained.[191] Keratocytes and schistocytes in blood of Fe-deficient animals is evidence of this pathologic process.
2. When Fe deficiency develops in domestic mammals, reticulocytosis is usually present but less than expected for the degree of anemia (release of reticulocytes is limited). Developing RBCs may become RNA depleted during their prolonged maturation prior to release from the marrow. Greater degrees of reticulocytosis have been reported, sometimes without information regarding prior Fe supplementation or transfusion.
3. Clinical data supporting that an anemia is due to chronic external blood loss:
   a. Melena, heme-positive urine or feces, or intestinal nematode ova, fleas, or other parasites (frank hemorrhage is typically not present)



A. Compensatory erythropoiesis prevents the development of anemia during weeks to months of blood and Fe loss.

B. As Fe deficiency develops, effective erythropoiesis diminishes and mild anemia develops.



C. When Fe is deficient, there is decreased effective erythropoiesis and more mitoses in Hgb-deficient cells resulting in release of microcytic hypochromic erythrocytes.

Figure 3.19. RBC kinetics of chronic blood loss that results in Fe deficiency.

A. Initially, there is a continual loss of small quantities of blood over weeks to months. Anemia does not develop as long as compensatory increased erythropoiesis (using stored Fe) replaces lost RBCs.

B. After prolonged blood loss, Fe deficiency develops (decreased total body Fe). When Fe deficiency is severe enough, effective erythropoiesis decreases enough that the compensation for blood loss is inadequate, and thus anemia ensues. Late-stage erythroid precursors remain longer in marrow and increase in number, but final maturation and release are impaired. Concurrently, the RBC life span decreases because of increased RBC membrane fragility. Fe deficiency affects many organs before anemia is present.

C. Microcytic and hypochromic RBCs result from the defective heme synthesis caused by Fe deficiency; Fe incorporation into heme for Hgb formation is decreased. While attempting to reach optimal cytoplasmic Hgb concentration, erythroid precursors are thought to undergo additional mitoses, so microcytes are formed (microcytic normochromic anemia). With severe Fe depletion, precursors are eventually unable to reach optimal cytoplasmic Hgb concentration, and then hypochromic cells may be formed (microcytic hypochromic anemia). Visible hypochromasia can be marked in dogs, ruminants, and camelids but is not typical of horses and cats.

b. Poorly regenerative anemia or nonregenerative anemia that is microcytic normochromic or microcytic hypochromic
c. Decreased CHr and MCVr[84,192]
d. Marrow (rarely assessed): erythroid hyperplasia but ineffective erythropoiesis because of maturation defects; hemosiderin absent
e. Mild to moderate hypoproteinemia
f. Hypoferremia, decreased total body Fe (depleted storage sites), and decreased serum [ferritin]
g. A history of frequent blood donations

4. Young animals are more prone to Fe-deficiency anemia than are mature animals because they have little stored Fe, consume less Fe on a milk diet, and require a large amount of Fe during growth and RBC production.

HEMOLYTIC ANEMIAS

I. Concepts and classifications
   A. Hemolysis (erythrolysis) is RBC necrosis and occurs at the end of every RBC's life. When the rate of in vivo hemolysis increases, then it is a pathologic state. Pathologic hemolysis (just called hemolysis) may be defined as an increased rate of RBC destruction that decreases RBC life span.
   B. Extravascular versus intravascular hemolysis
      1. Major differences
         a. With intravascular hemolysis, RBC destruction occurs in the blood, within blood vessels or heart, not including phagocytosis by tissue macrophages while RBCs pass through sinusoids of the spleen, liver, or bone marrow. Intravascular hemolysis is clinically recognized when it causes hemoglobinemia and hemoglobinuria. Decreased [haptoglobin] is also a marker for intravascular hemolysis in people, but [haptoglobin] is typically increased in dogs with hemolytic anemias,[193] despite mediating Hgb clearance via Hgb-haptoglobin complexes.[194] This may relate to concurrent inflammation and increased haptoglobin production as a positive acute-phase protein, as well as glucocorticoid-induced increases in some cases.
         b. With extravascular hemolysis, destruction of circulating RBCs occurs in macrophages near venular sinusoids of the spleen, liver, and bone marrow; there is no hemorrhage. Macrophages also can attach to RBCs within blood by reaching through noncontinuous capillary walls and then binding, engulfing, and lysing them. Extravascular hemolysis does not cause hemoglobinemia or hemoglobinuria, but commonly causes hyperbilirubinemia, predominantly of Bu.
      2. Major features of each type of hemolytic disorder are listed in Table 3.11.
      3. Why differentiate intravascular hemolysis from extravascular hemolysis?
         a. Recognizing the consequences of each type of hemolysis promotes understanding of disease processes and how they vary among patients.
         b. Establishing a major site of RBC destruction may be a diagnostic clue; certain diseases typically cause extravascular hemolysis, whereas others typically cause intravascular hemolysis (Table 3.12).
         c. Differentiation may be helpful in determining prognosis and treatment. Intravascular hemolysis usually occurs with life-threatening diseases, so its presence suggests a poorer prognosis, and immediate treatment and management of the case are indicated.

Table 3.11. Common major features of intravascular and extravascular hemolytic disorders

Feature	Clinical intravascular hemolysis[a]	Mostly extravascular hemolysis[b]
Site of hemolysis	Within blood vessels or heart	Macrophages near sinusoids of spleen, liver, or marrow
Degree of direct RBC damage by the process or hemolytic agent	Marked	Mild to marked
Severity of anemia	Marked (or rapid development)	Mild to marked
Onset of illness	Hours to days	Days to weeks
Reticulocytosis	Usually after initial presentation	Usually at initial presentation
Hemoglobinemia	Yes, but may not be grossly visible	No
Hemoglobinuria	Yes	No
Hyperbilirubinemia	No or yes[c]	Usually at presentation; Bu > Bc
Bilirubinuria	No or yes[c]	Usually at presentation

[a] Clinical intravascular hemolysis is recognized by finding pathologic hemoglobinemia or pathologic hemoglobinuria. Concurrent extravascular hemolysis is likely.

[b] During these disorders, intravascular hemolysis may be occurring but not severely enough to cause hemoglobinemia or hemoglobinuria.

[c] Soon after the onset of intravascular hemolysis, hyperbilirubinemia and bilirubinuria are probably not present. With time, increased macrophage degradation of Hgb from intravascular hemolysis may contribute to excess bilirubin formation and thus hyperbilirubinemia or bilirubinuria. Hyperbilirubinemia and bilirubinuria are expected when there is concurrent extravascular hemolysis of sufficient duration and severity.

d. However, both processes are often present concurrently, and extravascular hemolysis is expected to accompany intravascular hemolysis. Also, disorders may switch from predominantly one to predominantly the other.

C. Thorough examination of RBCs in a blood film is an essential diagnostic procedure for suspected or confirmed hemolytic anemias. A well-made and well-stained blood smear and a good microscope with a 100× oil objective are needed for such examinations. One may see organisms or other clues of a hemolytic process.
   1. Organisms: *Mycoplasma*, *Anaplasma*, *Babesia*, and *Theileria*
   2. Other clues of a hemolytic process: spherocytes, ghost cells, Heinz bodies, eccentrocytes, pyknocytes, schistocytes, keratocytes, and acanthocytes

D. Hemolytic icterus (jaundice) (Figure 3.20)
   1. Pathologic hemolysis leads to increased Hgb degradation, thus increased bilirubin formation, and perhaps development of icterus (serum, plasma, other body fluid, or tissues visibly colored by bilirubin). Icterus may develop in animals with predominantly intravascular or predominantly extravascular hemolytic disorders. In both forms, Hgb degradation increases, but the sites of RBC destruction differ. Icterus may develop in animals with intravascular hemolysis because of concurrent extravascular hemolysis.

Table 3.12. Hemolytic disorders and conditions
Immune-mediated hemolysis not associated with infections
Idiopathic[a] (includes autoimmune)*
Drug induced[a]
Alloimmune:[a] neonatal isoerythrolysis, incompatible blood transfusion reactions
Infectious hemolytic anemias[a]
Hemotropic *Mycoplasma* spp.*
Anaplasma spp.*
Leptospira spp.[b]
Clostridium spp. causing RBC membrane damage by phospholipases
Bacillary hemoglobinuria (*C. haemolyticum* or *C. novyii*)[a]
Yellow lamb disease (*C. perfringens*, type A)
Clostridial infections in horses (*C. perfringens*, type A)
EIAV[a,b]
Protozoal infections of RBCs: *Babesia* spp.,[a,b]* *Cytauxzoon* spp. (some species or strains, not typically *C. felis*), *Theileria* spp.,[b] and *Rangelia vitalii*
Trypanosoma spp.[b]
Secondary to *Rhodococcus equi* pneumonia (horses)
Erythrocytic metabolic defects (acquired or inherited)
Oxidative damage*
Heinz body hemolysis[a]
Eccentrocytic hemolysis (acquired or inherited)[a]
Defects in ATP generation
Pyruvate kinase deficiency
Phosphofructokinase deficiency[a]
Hypophosphatemic hemolysis[b]
L-Sorbose intoxication
Defects in heme synthesis: Bovine or feline congenital erythropoietic porphyria
RBC fragmentation in blood: intravascular coagulation (localized or disseminated),* vasculitis,* hemangiosarcoma, caval syndrome (dirofilariasis),[a] cardiac valve disease
Hemolytic anemia of other or unknown pathogeneses
Idiopathic nonspherocytic hemolytic disorders with increased osmotic fragility
Hereditary nonspherocytic hemolytic anemia of beagles
Idiopathic hemolytic anemia of cats (Abyssinian, Somali, other)
Hypoosomolar hemolysis
Envenomation (snakes, spiders, insects)
Hemophagocytic histiocytic sarcoma
Hemophagocytic syndrome
Heparin-induced hemolysis (horses)
Hemolytic syndrome in horses with hepatic disease[a,b]

* A relatively common disorder, condition, or mechanism (varies by location)

[a] Hemoglobinuria or hemoglobinemia may be present because of marked intravascular hemolysis.

[b] Other mechanisms may also contribute to anemia.

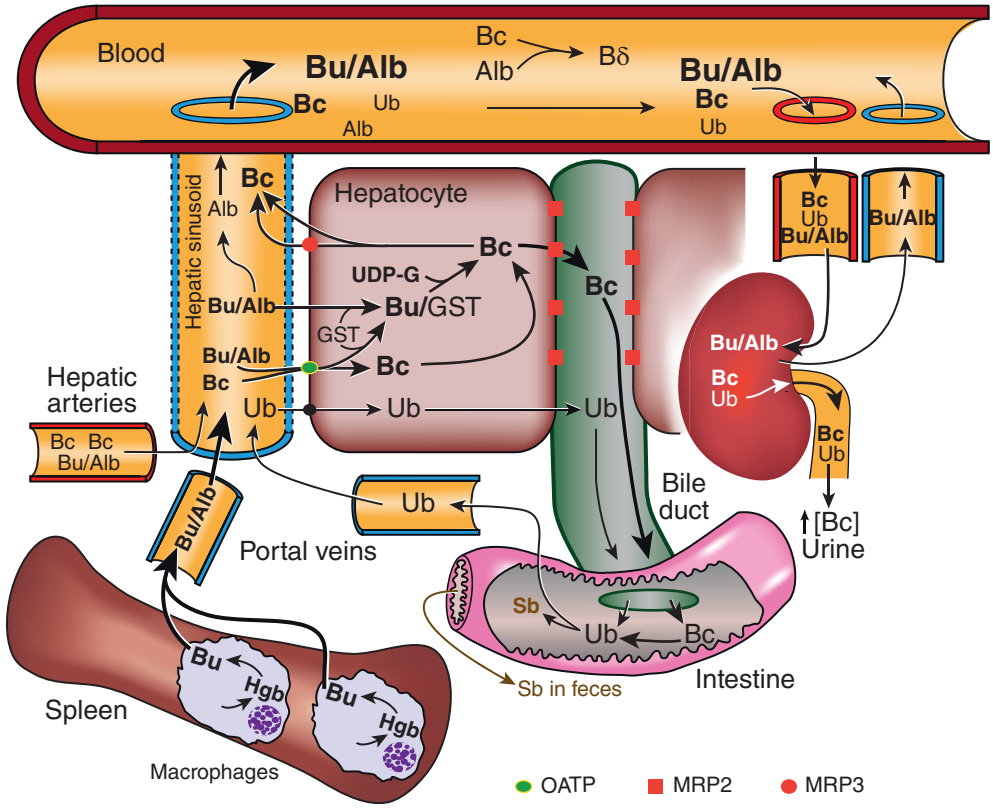


Figure 3.20. Hemolytic icterus (compare to Figure 3.3). Accelerated destruction of RBCs in macrophages of spleen (also liver and marrow) increases the production and delivery of Bu to hepatocytes. If the rate of Bu formation exceeds the liver's ability to clear Bu from plasma, hyperbilirubinemia with increased [Bu] develops. Increased delivery of Bu to hepatocytes also increases Bc formation and biliary excretion. If Bc formation exceeds Bc transport to canaliculi via MRP2, then excess hepatocellular Bc enters sinusoidal plasma by MRP3 or diffusion. The increased [Bc] in plasma increases the Bc excretion in urine (bilirubinuria). If there is persistently increased plasma [Bc], there may be increased formation of Bδ, Bc covalently bound to Alb (see the Bilirubin Concentration section in Chapter 13).
Bu/Alb, Bu associated with albumin; OATP, organic anion transporting polypeptide; MRP2 or 3, multidrug resistance-associated protein 2 or 3; Sb, stercobilinogen; Ub, urobilinogen; UDP-G, uridine diphosphoglucuronide

2. Hemolytic hyperbilirubinemia occurs when Bu travels through the blood from tissue macrophages to the liver. Except for uncommon situations, the capacity for Bu uptake greatly exceeds that of bilirubin excretion, so uptake is not rate limiting. If Bu formation exceeds an animal's ability to excrete it into the bile as Bc, hyperbilirubinemia develops. If the capacity of the liver for Bu uptake, conjugation, and excretion are not exceeded, serum [bilirubin] may remain WRI even though pathologic hemolysis is present.
3. The rate-limiting step in bilirubin excretion is the transport of Bc to bile canaliculi. If the transport maximum is reached, and likely to some degree prior to that, Bc is pumped from hepatocytes to plasma and either excreted in urine or taken back up by hepatocytes.

4. Bu and Bc compete for the same receptors on hepatocytes. Thus, major increases in [Bu] impair reuptake of any Bc that is pumped or diffuses to sinusoids, so both forms increase in plasma. However, as in human patients,[195] [Bu] > [Bc] in the vast majority of domestic mammals with icterus of hemolytic origin. In longer-standing hemolytic disorders (1 wk or longer), the [Bc] may approach the [Bu] or exceed it if there is hepatic damage caused by hypoxia or other insults.

E. Bilirubinuria (bilirubin in urine)
1. Bc is small, H_2O soluble, and not protein bound, so it passes through the glomerular filtration barrier and is not resorbed (it has a low renal threshold).
2. Bu is H_2O insoluble and bound with albumin in plasma, so little or no Bu passes the glomerular filtration barrier without glomerular disease.
3. Some bilirubin is present in the urine of healthy dogs. The bilirubin may be Bc (the urine bilirubin assay reportedly detects Bc but not Bu), but many apparently healthy dogs have mild albuminuria, and thus the detected bilirubin might include Bu bound to albumin, or canine kidneys may convert Bu to Bc and excrete Bc in the urine.
4. Bilirubinuria usually occurs before clinical hyperbilirubinemia (icterus) because Bc is excreted in urine as soon as its concentration in blood starts to increase, whereas clinical icterus is usually not recognized until serum [bilirubin] reaches at least 1.5 mg/dL. However, bilirubinuria may be missed because of false-negative urine bilirubin results.

F. Urobilinogenuria: Renal urobilinogen excretion increases in hemolytic anemias because more heme and heme degradation products are being processed.

G. Hemolytic hemoglobinemia and hemoglobinuria (Figure 3.21)
1. A key to recognizing intravascular hemolysis is differentiating the pathologic causes of red, brown, or black urine. These are primarily hemoglobinuria, hematuria, and myoglobinuria. These pigmenturias can typically be classified by criteria listed in Table 3.13. Other causes of pigmenturia are described in Physical Examination of Urine, Section I.C. in Chapter 8.
2. Hgb nephropathy is a pathologic state seen in intravascular hemolytic disorders characterized by proximal tubular degeneration that may result in acute renal insufficiency. Hgb or metHgb casts may be present in renal tubules and/or urine. Evidence suggests that hemolysis-induced renal injury requires such contributing factors as hypovolemia and aciduria. Mechanisms of injury may include heme-induced oxidation, cast-induced tubular obstruction, and vasoconstriction from Hgb scavenging of nitric oxide, a vasodilator.

II. Hemolytic disorders (Tables 3.12 and 3.14)

A. Immune-mediated hemolytic anemias (not associated with infections)
1. Concepts
a. Immunologic hemolytic anemias occur when an animal's immune system produces antibodies that bind directly or indirectly to its own RBCs (ESAIg) and lead to RBC destruction. The process may be initiated by a defective immune system, defective RBCs, or adsorbed antigens from drugs, infectious agents, or possibly neoplasms. Factors that initiate the process are usually not known. Associated disorders may be coincidental or causative.
b. Hemolysis may occur through three major processes as described in Figure 3.22: phagocytosis, complement-mediated lysis, or fragility-mediated lysis.
c. Clinical evidence may suggest the presence of intravascular hemolysis, extravascular hemolysis, or both. ESAIg molecules that mediate immune hemolysis may be IgG, IgM, or IgA. Extravascular hemolysis occurs when RBCs coated with

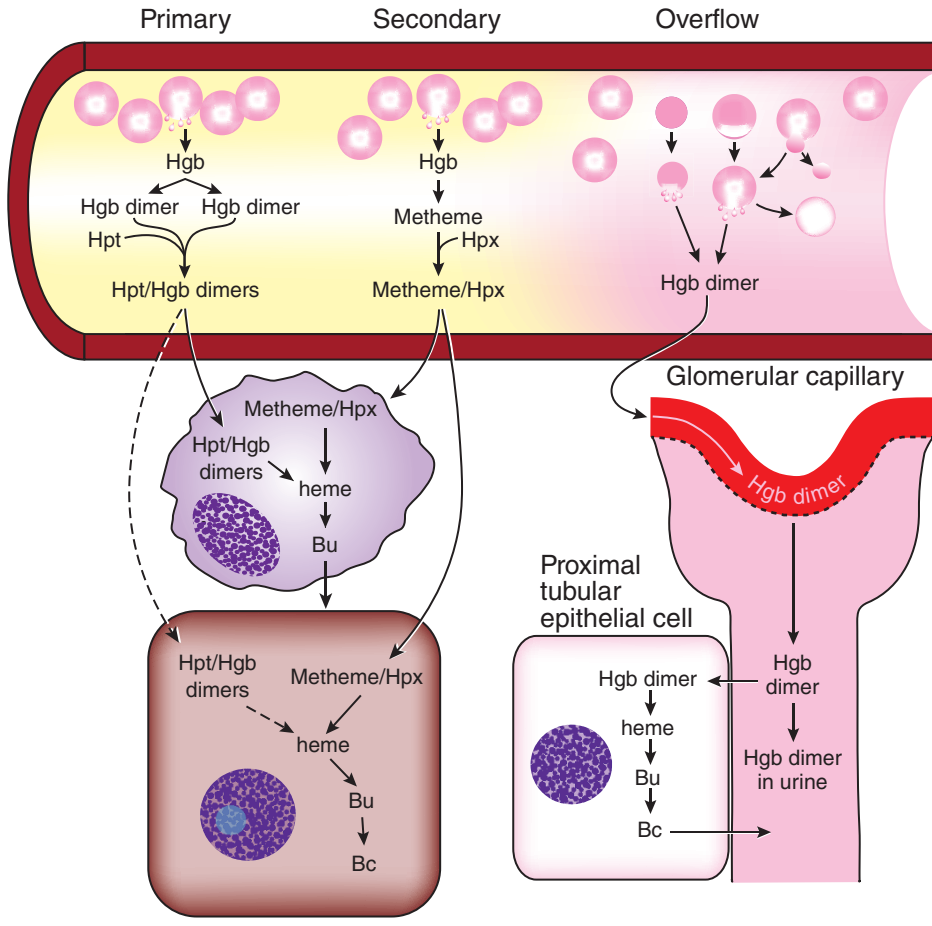


Figure 3.21. Pathogenesis of hemoglobinemia and hemoglobinuria during intravascular hemolysis.

- Physiological processes that conserve Fe and limit oxidative damage by Hgb released during intravascular hemolysis can be divided into primary and secondary systems. These systems are not saturated during health, and thus free Hgb is not seen in health. In pathologic states, saturation of the primary and secondary systems leads to free hemoglobinemia and hemoglobinuria (loss of Hgb and Fe). Several disorders cause intravascular hemolysis, including those that create spherocytes, eccentrocytes, Heinz bodies, or ghost RBCs.
- Primary (most important) scavenger system for Hgb and Fe conservation
  - Intravascular RBC damage or death may release Hgb to plasma. Hgb tetramers split into dimers which immediately bind Hpt (if available), thus impeding passage of Hgb through the glomerular filtration barrier; *haptoglobin* is from the Greek *haptein*, "to bind" + (hemo)globin. The Hpt/Hgb complexes are thought to be cleared from plasma mostly by hepatic macrophages (via CD163 receptors), but hepatocytes may also play a role.[196-198] Hgb is degraded to Bu, Fe^{3+}, and amino acids.
  - Plasma [Hgb] or [Hgb/Hpt] may be great enough to cause the plasma to be pink, thus hemoglobinemia is recognized. Plasma is pink when plasma [Hgb] is as low as 50 mg/dL.
- Secondary system of Fe conservation (increased importance if plasma [Hpt] decreases)
  - Continued or more severe intravascular RBC damage or death causes release of more Hgb to plasma. The plasma Hgb may oxidize to form metHgb, which dissociates and releases metheme and globin. Metheme binds to Hpx to form a metheme/Hpx complex.[199]

Table 3.13. Differential features of hematuria, hemoglobinuria, and myoglobinuria

	Hematuria	Hemoglobinuria	Myoglobinuria
Hct	WRI[a]	Decreased	WRI
Plasma color	Not pink to red[b]	Pink to red	Not pink to red[b]
Urine color	Pink to red[c]	Pink to red[c]	Pink to red[c]
Urine heme reaction[c,d]	Positive	Positive	Positive
RBCs in urine sediment	Yes[e]	No[f]	No[f]
Information to support muscle damage[g]	No	No	Yes

[a] Unless there is concurrent disease or an associated disorder that causes extensive hemorrhage
[b] Unless there is concurrent in vivo or in vitro hemolysis
[c] Heme typically is red but may become brown to black (with degradation or oxidation) because of metheme formation. Metheme can produce a positive urine heme reaction.
[d] The ammonium sulfate precipitation test does not reliably differentiate Hgb from myoglobin (see Chemical Examination of Urine, Section VI.C.3, in Chapter 8).
[e] RBCs may lyse after they enter urine (especially in dilute) and thus may not be seen in a urine sediment examination.
[f] Hematuria may be present because of another pathologic process or due to sample collection.
[g] Information may include historical or physical evidence of muscle damage (e.g., stiffness or trauma) or increased serum creatine kinase activity.

immunoglobulins are engulfed by macrophages. If the immunoglobulins fix complement, the membrane attack complex (C5b–9) may form membrane pores and cause intravascular hemolysis. This complex consists of complement proteins C5b, C6, C7, C8, and multiple copies of C9, which form a ring in the membrane and enable free diffusion of molecules into and out of the cell. Variations in the types and properties of pathogenic antibodies create variations in clinical manifestations.

Figure 3.21. *continued*

- Metheme/Hpx complexes bind receptors (CD91) and enter hepatocytes and macrophages, where the complexes are degraded to Bu, amino acids, and Fe^{3+}.[199,200] Bu formed in hepatic macrophages is released and taken up by hepatocytes; this Bu and that formed in hepatocytes is conjugated to Bc for excretion.
- In people and nonhuman primates, metheme also binds to albumin, and the metheme/albumin complex enters hepatocytes so that Fe can be conserved. However, albumin molecules of dogs, cattle, horses, and other domestic mammals do not bind metheme, and thus this pathway is not functional in those animals.[201]

• Overflow hemoglobinuria occurs with overwhelming intravascular hemolysis when the Fe conservation pathways are saturated.
- If the rate of hemolysis exceeds the ability of Hgb-binding proteins to conserve Fe, free Hgb dimers accumulate in plasma (hemoglobinemia).
- Hgb dimers pass through the glomerular filtration barrier and are excreted in the urine (hemoglobinuria) if the resorption capacity of proximal tubule epithelial cells is exceeded. Resorbed Hgb can be degraded by renal tubular epithelial cells, conjugated to Bc, and excreted in the urine.

Hpt, haptoglobin; Hpx, hemopexin

Table 3.14. Recognized hemolytic disorders of dogs, cats, horses, and cattle

Type of anemia	Dogs	Cats	Horses	Cattle
Immune-mediated, noninfectious	IMHA Drug-induced NI Transfusion	IMHA NI Transfusion	IMHA Drug-induced NI Transfusion	IMHA Transfusion
Infectious	*Mycoplasma* *Babesia* *Rangelia*	*Mycoplasma* *Cytauxzoon*[a]	*Babesia* *Leptospira* Equine infectious anemia *Clostridium* *Theileria*	*Mycoplasma* *Anaplasma* *Babesia* *Theileria* *Leptospira* *Clostridium* *Trypanosoma*
Metabolic	Heinz body Eccentrocytic PK deficiency PFK deficiency Hypophosphatemia L-Sorbose toxicosis	Heinz body PK deficiency Erythropoietic porphyria	Heinz body Eccentrocytic	Heinz body Hypophosphatemia Congenital erythropoietic porphyria
Traumatic	Angiopathy	Angiopathy	Angiopathy	—
Other	Envenomation Hemophagocytic histiocytic sarcoma Hereditary nonspherocytic	Idiopathic osmotic fragility syndrome	Heparin-induced Hepatic disease	Hypoosmolar

[a] Hemolysis may be a minor contributor to the anemia with *C. felis* infections and may be more significant with genetically distinct species of *Cytauxzoon* (see Nonregenerative Anemias, Section II.B.2).
Note: Hemolytic disorders seen in alpacas and llamas include *Mycoplasma*-induced and Heinz body–associated anemias.

d. The DAT (tube, plate, or flow cytometric methods) may be used to detect ESAIg or C3 on a patient's RBCs (see Methods to Detect RBC Surface Antibody or Complement).

e. To understand some of the clinical manifestations and laboratory findings in immune-mediated hemolytic anemias, the major features of warm and cold antibodies need to be understood.

(1) Warm antibodies (usually IgG) are more common than pathologic cold antibodies and have maximal binding activity at body temperature (37–39 °C). They may bind to RBCs at room temperatures and thus can cause autoagglutination in samples at routine handling temperatures.

(2) Cold antibodies (usually IgM), have maximal binding activity at below body temperature (often 4–20 °C). If they bind at high enough temperatures, they may bind RBCs in vivo while passing through cool extremities, and thus they can activate complement and initiate immune hemolysis. They typically cause autoagglutination as RBCs cool during or after collection. Not all cold

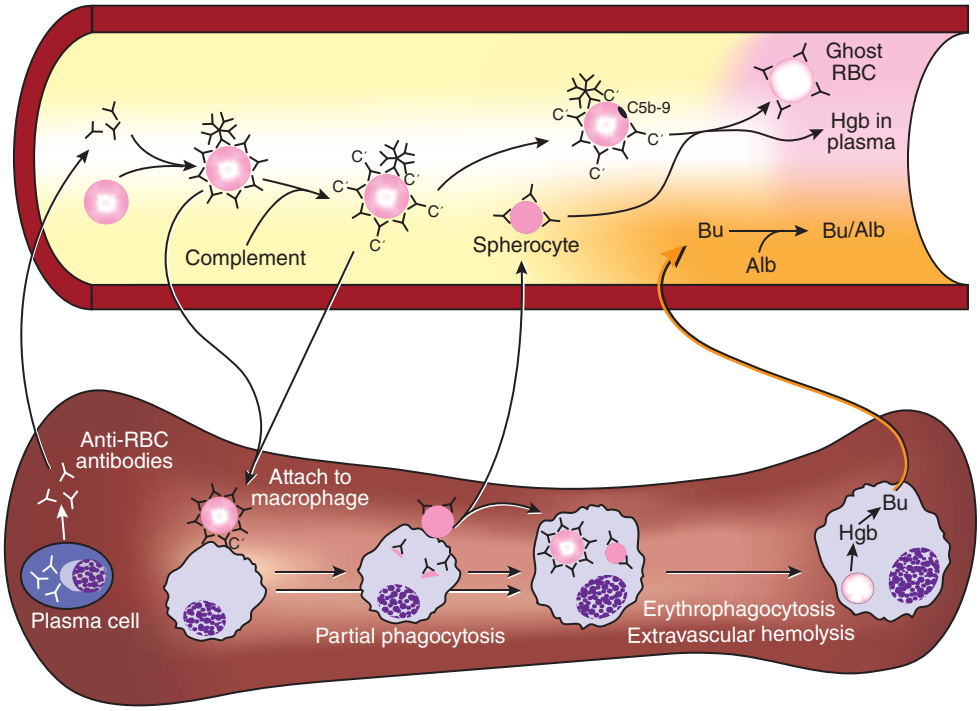


Figure 3.22. Pathogenesis of immune-mediated hemolysis.

- Antibodies (mostly IgG and IgM) to RBC-associated antigens are formed by plasma cells or B lymphocytes.
- Antibodies bind to epitopes on circulating RBCs. Some antibodies, especially IgM, activate complement leading to C3 (C') binding to ESAIg or RBCs.
- Activation of the complement cascade can lead to intravascular hemolysis via membrane attack complex (C5b–9) pores. Damaged ghosts cells are removed from circulation by macrophages (not shown). These processes contribute to anemia.
- RBCs coated with IgG and/or C3 attach to macrophages via Fcγ receptors for IgG, complement receptors, or both.
- If macrophages digest and remove RBC membrane without engulfing the cell, spherocytes may form and return to circulation.
- Spherocytes may undergo intravascular hemolysis because of their fragility. Fragility or complement-mediated hemolysis can create hemoglobinemia.
- Macrophages may completely engulf RBCs, including spherocytes and damaged RBCs, thus contributing to anemia.
- Macrophages degrade engulfed RBCs within phagolysosomes, and convert heme to Bu, thus extravascular hemolysis contributes to hyperbilirubinemia and icterus.

C5b–9, complex of complement factors 5b through 9; Fcγ, crystallizable fragment of IgG (the base of the Y-shaped IgG)

antibodies are pathologic in vivo, even if they cause autoagglutination in vitro,[56] and they may or may not be associated with other pathologic antibodies.

2. Disorders
   a. IMHA
      (1) IMHA is the most common hemolytic anemia of dogs. It is also occasionally found in cats, horses, and cattle.

(2) Factors initiating immune hemolysis are unknown, as are specific antibody targets.[202] IMHA caused by an underlying disorder (e.g., infection, neoplasm, drug, systemic lupus erythematosus) is secondary IMHA while that occurring without an associated condition is idiopathic IMHA and may be primary IMHA (autoimmune hemolytic anemia with targeting of autoantigens). Truly autoimmune RBC destruction results from immune responses to normally occurring self-epitopes. When the epitopes are from foreign antigens or alloantigens, the body's own cells are destroyed, but the process is not strictly speaking autoimmune. Typically, it is assumed that IMHA is an autoimmune process when causes of immune hemolysis are not found.

(3) A working diagnosis of IMHA is based on a constellation of objective and subjective findings, each of which alone is not specific for IMHA, each of which is subject to error, and most of which can be absent in IMHA:[203]

(a) Anemia (usually moderate to marked)

(b) Evidence of intravascular or extravascular hemolysis: hemoglobinemia, hemoglobinuria, ghost cells, hyperbilirubinemia (mostly unconjugated), hyperbilirubinuria (dogs), or bilirubinuria

(c) Evidence of an immune-mediated targeting of RBCs: spherocytosis, autoagglutination, or DAT positivity

(d) Exclusion of other possible explanations for the findings

(4) Classic laboratory features of IMHA: regenerative anemia (mild to severe), icterus, possibly hemoglobinuria, spherocytosis (difficult to recognize in species other than dogs), positive DAT, inflammatory leukogram including neutrophilia with regenerative left shift and monocytosis, and no evidence of other causes of anemia

(5) Exceptions to classic features

(a) Anemia may be nonregenerative if present < 2–3 d, if antibodies also attack erythroid precursors (e.g., mixed IMHA and PIMA or pure red cell aplasia), or if another disease process interferes with erythropoiesis.

(b) Evidence of extravascular hemolysis (icterus, bilirubinuria, etc.) may not be present if the rate of RBC destruction is only slightly increased or if accelerated hemolysis is brief.

(c) Spherocytosis may not be present because spherocytes may not accumulate in blood if their rate of formation is less than the rate of their removal.

(d) The results of the DAT may be negative due to a low density of ESAIg, presence of an ESAIg isotype not detected by the assay, or technical problems including prozone reaction, poor reagents, or poor sample.

(6) Other laboratory or diagnostic features of some IMHA cases: RBC autoagglutination, ghost RBCs, thrombocytopenia or other evidence of a prothrombotic state, and splenomegaly

b. Drug-induced IMHA

(1) Drugs may induce immune-mediated hemolysis through multiple mechanisms, the understanding of which is incomplete.[204]

(a) Some drugs (e.g., penicillin) or their metabolites are *haptens*, which are antigenic but not immunogenic until they bind covalently or weakly with another molecule, usually a protein. Binding to RBCs may stimulate production of antibodies that bind to the drug or metabolite itself, a combined drug and membrane protein neoantigen, and/or primarily

membrane proteins. The first two are *drug-dependent antibodies*, binding only in the presence of the drug. The third may include *drug-independent antibodies* that bind to cell membranes in the absence of drug, thus acting like autoantibodies.

(b) Some drugs (e.g., methyldopa and procainamide in people) cause the formation of autoantibodies that bind to RBCs in the absence of the drug. This may occur through effects of the drugs on the immune system.

(2) Drugs reported to initiate IMHA in domestic mammals include penicillin in horses,[205,206] propylthiouracil in cats,[207] cephalosporins in dogs (suprapharmacologic doses),[208] trimethoprim-sulfamethoxazole in horses,[209] levamisole in dogs,[210] and pirimicarb in dogs.[211] Over 100 drugs are reported to cause IMHA in people.

c. Vaccine-induced IMHA: Some clinicians have associated the onset of immune-mediated anemia in dogs with recent vaccinations, but little data have been generated to support a cause-and-effect relationship.[203]

d. Alloimmune hemolysis

(1) NI (or NIE), also known as hemolytic disease of the newborn

(a) *Isoerythrolysis* (iso = same; erythrolysis = hemolysis) refers to hemolysis caused by immunization with RBC antigens from another member of the same (iso-) species. This condition can also be called neonatal alloimmune hemolytic anemia because it is commonly caused by exposure to *alloantigens*, i.e., antigenic variations in a molecule that is typically present in all members of a species.

(b) In NI, ingested maternal colostral antibodies are absorbed by the intestine, enter blood, and then attach to the neonate's paternally derived RBC surface antigens. The antibody-coated RBCs are then lysed by macrophages or complement.

(c) In cats, NI occurs when a queen with type B blood mates with a type A tom and passes enough of her anti-A alloantibodies to her type A or type AB kittens via colostrum. Blood type B is uncommon in American domestic shorthair cats (< 5 %) but reported in 20–30 % of cats in New Zealand and Australia. The highest prevalence (about 30–40 %) is found in British shorthair, Devon rex, and Cornish rex cats. Blood type AB is rarest, but relatively common in ragdolls (20 %) and reported in as many as 5–10 % of cats in some countries. Anti-B alloantibodies of type A cats typically have weak activity and thus are not associated with NI. Type AB cats do not have alloantibodies.[212]

(d) In horses, NI may occur if a mare is negative for Aa or Qa antigens, she has acquired alloantibodies against such antigens during prior pregnancies (perhaps the current one) or transfusions, she has produced colostrum containing these alloantibodies, and her foal is positive for the Aa or Qa alloantigen (paternally derived). Other alloantigens typically induce an alloantibody response that is too weak to cause isoerythrolysis,[213] although NI has been associated with several, including the Ab, Ac, Qb, Qc, Db, Dg, Pa, Pb, Ua, and possibly Ka alloantigens.[25,214–216] Suspected nonspecific hemolytic alloantibodies reactive with RBCs from NI foals and an array of test horses but unreactive with RBCs from the foals' mares have been reported but not characterized.[216] In one study involving 799 mares,

anti-RBC antibodies were detected via hemolytic or agglutination tests in 20 % of the standardbred and 10 % of the thoroughbred mares. However, most of the antibodies were anti-Ca; only 1 % of thoroughbreds and 2 % of standardbreds had either anti-Aa or anti-Qa antibodies.[217] Anti-Ca antibodies do not appear to mediate NI.

(e) Mules, from a donkey sire and a horse dam, are more likely than horses to have NI because all horses appear to lack an undefined immunogenic donkey factor.

(f) In dogs, NI may occur if a DEA 1–negative bitch develops anti-(DEA 1) antibodies after being transfused with DEA 1–positive blood, and then passes those antibodies on to a DEA 1–positive puppy via colostrum. Dogs are not known to have clinically significant naturally occurring alloantibodies to DEA 1, and other acquired alloantibodies are not known to cause NI.

(2) Immune-mediated hemolysis secondary to incompatible blood transfusions

(a) Transfused donor RBCs are attacked by recipient (patient) circulating anti-RBC antibodies. Transfused RBCs are recognized as foreign when the donor and recipient have different antigens on their RBC membranes. These are alloantigens causing alloimmune hemolytic anemia.

(b) The alloantibodies that cause hemolysis are the same as those that cause NI. In horses, the alloantibodies are acquired antibodies that develop during pregnancy, parturition, or after whole blood transfusions. In cats, the antibodies are naturally occurring alloantibodies. In dogs, pregnancy has not been shown to induce alloantibodies, and naturally occurring alloantibodies of clinical significance are rare.[218]

(c) Blood typing and crossmatching are used to reduce immune-mediated hemolytic transfusion reactions (see the Blood Typing and Crossmatching section).

(3) Early attempts to develop a vaccine for bovine anaplasmosis resulted in production of anti-RBC antibodies because of bovine RBC stroma within the vaccine.[219] These antibodies were passed in colostrum and led to NI.[220] Purification of blood-derived vaccines reduced the stroma problem, but the vaccines became unavailable during the 1990s.[219]

B. Infectious hemolytic anemias

1. Infections may be mixed, particularly with tick-borne diseases.
2. Hemotropic mycoplasma species

a. These are obligate extracellular bacteria that lack a cell wall around their cell membrane. Multiple mycoplasma species may infect an animal at one time; molecular techniques are necessary to confirm speciation.

b. Feline hemic *Mycoplasma* spp. (basonym, *Haemobartonella* spp.)[221] causing feline infectious anemia are present worldwide.

(1) *Mycoplasma haemofelis*: This bacterium was originally called the Ohio or Florida strain of *Haemobartonella felis*. It is larger than the other feline hemotropic *Mycoplasma* spp., and more pathogenic. Infected cats may have concurrent predisposing retroviral infections.

(2) "*Candidatus* Mycoplasma haemominutum": This bacterium was called the smaller form or California species of *Haemobartonella felis*. It is more common than the other feline hemotropic mycoplasmas, but it is less pathogenic.

(3) "*Candidatus* Mycoplasma turicensis":[222] This bacterium is thought to be of low pathogenicity in the absence of concurrent disease or immunosuppression. Bacteremia tends to be mild and short, so organisms are not expected to be seen in blood smears.

(4) Molecular testing in prevalence studies have detected "*Candidatus* Mycoplasma haematoparvum" and "*Candidatus* Mycoplasma haematoparvum"-like organisms in cats, but their role in disease is unknown.

(5) Bacteremia is usually present during hemolysis, but it may suddenly disappear (within hours).

c. Canine hemic *Mycoplasma* spp.

(1) *Mycoplasma haemocanis* (basonym, *Haemobartonella canis*): Bacteremia is usually restricted to splenectomized or otherwise immunocompromised dogs.

(2) "*Candidatus* Mycoplasma haematoparvum": This small bacterium was visible microscopically in a splenectomized dog,[223] and genetic analysis indicates it is more closely related to "*Candidatus* Mycoplasma haemominutum" than to *Mycoplasma haemocanis*.[224] By PCR, it was detected in about one-third of pit bull–type dogs, unassociated with anemia.[225]

d. Bovine hemic *Mycoplasma* spp. (basonym, *Eperythrozoon*)

(1) *Mycoplasma wenyonii*: Bacteremia and anemia are usually mild, but marked bacteremia and severe anemia can occur, particularly in young or immunocompromised (including splenectomized) cattle, in which case icterus and hemoglobinuria may be present. Other reported clinical signs include hindlimb edema, scrotal edema, swollen teats, fever, lymphadenopathy, decreased milk production, and reproductive problems. Immune-mediated vasculitis may promote edema.

(2) "*Candidatus* Mycoplasma haematobovis (haemobos)": Subclinical infections appear to be most common, but infection has been associated with anemia, decreased milk production, lameness, and infertility. Co-infections with *M. wenyonii* occur.

e. Llama and alpaca hemic *Mycoplasma* spp. (previously called *Eperythrozoon*): "*Candidatus* Mycoplasma haemolamae" usually causes subclinical infections that are not associated with anemia, but marked bacteremia can occur, and bacteremia has been associated with mild to marked anemias that may or may not be regenerative and could be caused by concurrent disease.

f. Hemotropic *Mycoplasma* spp. (including *M. ovis*) have been detected by PCR in horses from multiple continents, but reports are rare and their contribution to disease is not clear.[226]

g. Hemotropic *Mycoplasma* spp. (basonym, *Eperythrozoon*) infect pigs, sheep, and goats and may cause hemolytic anemia.

h. Pathogeneses of these hemolytic anemias are thought to involve antibodies binding to the parasitized RBCs, either because of bound parasite antigens or RBC antigens exposed on altered membranes.

i. Major laboratory findings in patients with clinical hemolytic anemias caused by *Mycoplasma* spp. include *Mycoplasma* spp. on RBCs in films of fresh blood. Bacteria are most numerous when Hct is falling. Organisms may detach from RBCs in vitro, so organisms are best detected in blood films made soon after blood collection. Stain precipitate adherent to RBCs may be mistaken for *Mycoplasma* spp.

(1) Laboratory findings may include moderate to severe anemia, reticulocytosis with polychromasia, mild to moderate hyperbilirubinemia and bilirubinuria, a positive DAT, spherocytosis (dogs), autoagglutination, PCR positivity for *Mycoplasma* spp., and hypoglycemia with marked bacteremia in camelids, cattle, sheep, and pigs (especially when young).

(2) Many animals are subclinical carriers of *Mycoplasma* spp.; positive PCR results do not indicate clinical disease without other supporting findings, and microscopic examination of blood cannot be used to confirm negativity.

(3) Findings may vary when associated with underlying or concurrent disease (*Mycoplasma* spp. are opportunistic bacteria).

3. *Anaplasma* spp. (RBC-infecting anaplasmal species)
   a. *Anaplasma marginale* (cattle), *A. ovis* (sheep and goats), and the less pathogenic *A. centrale* (mostly cattle of South America, Africa, and the Middle East, but reported in Europe) are intracellular bacteria.
   b. *Anaplasma marginale* are most numerous when Hct is falling: 4–5 d later, it is difficult to find organisms. The bacterium forms an intracellular marginal body associated with the RBC membrane.
   c. Pathogenesis of anemia is mostly immune mediated. Antibodies bind to RBCs damaged by the anaplasmal infection and cause extravascular hemolysis.[227,228]
   d. Major laboratory findings are the presence of intracellular bacteria (marginal bodies) found early in clinical disease, moderate to severe anemia, reticulocytosis, increased polychromasia, often basophilic stippling, mild to marked hyperbilirubinemia and bilirubinuria, and PCR positivity for *Anaplasma* spp.
4. *Leptospira* spp.
   a. *Leptospira interrogans* serovars *pomona* and *icterohemorrhagica* do not infect RBCs, but vasculitis and infection of kidneys or liver can lead to a hemolytic state.
   b. *Leptospira* spp. infect most domestic animals and many wild animals, but the hemolytic state is seen primarily in calves, lambs, and pigs. Toxins of *Leptospira* spp. are not known to be in vivo hemolysins in dogs and cats.
   c. Pathogenesis of the anemia is not established but may involve an immunologic response associated with an IgM cold agglutinin[229,230] or leptospiral phospholipase activity.[231]
   d. Major laboratory findings: moderate to severe anemia, hemoglobinemia and hemoglobinuria, hyperbilirubinemia and bilirubinuria, neutrophilia, leptospiral spirochetes detected in urine or other fluids by direct dark-field microscopic examination, at least a fourfold increase in convalescent titer to either pomona or icterohemorrhagica serotypes, and PCR positivity for *Leptospira* spp.
5. *Clostridium* spp.
   a. *Clostridium haemolyticum* and *C. novyii* type D, the causes of bacillary hemoglobinuria in cattle and sheep (red water disease, Nevada red water)
      (1) *Clostridium haemolyticum* and *C. novyii* type D are inhabitants of soil and may remain dormant in cattle until anaerobic conditions promote their growth. The disease has been reported in many parts of the world. In the United States, bacillary hemoglobinuria is seen primarily in poorly draining areas of Gulf Coast and Western states. It has been associated with hepatic flukes.
      (2) Pathogenesis of anemia: RBC damage is caused primarily by a β toxin that has phospholipase C lecithinase activity, thus hydrolyzing phosphatidylcholine and causing lysis of RBCs. Other relatively minor hemolysins (lipase, proteinase, and another lecithinase) have been reported as products of *C. haemolyticum*.[232]

(3) Major laboratory findings: acute severe anemia, hemoglobinemia, and hemoglobinuria

(4) Confirmatory diagnostic findings: postmortem lesions, Gram-positive bacilli in tissues (liver and spleen) or fluids (blood and peritoneal fluid), *C. haemolyticum* cultured from liver, and phospholipase C activity (β toxin) detected in tissues

b. *Clostridium perfringens* type A (formerly *C. welchii*) in ruminants, causing yellow lamb disease in calves and lambs (also known as enterotoxemic jaundice or "the yellows")

(1) *Clostridium perfringens* type A is part of the intestinal microbiome, but multiplication has been associated with highly proteinaceous diets, overfeeding, and starchy foods. In the United States, yellow lamb disease is a lethal disease that occurs in nursing spring lambs in northern California and Oregon.[233] It also occurs in lambs and calves of other regions (e.g., Canada, Australia, New Zealand, and South Africa).

(2) Pathogenesis of anemia: Phospholipase C (EC 3.1.4.3), the α toxin produced by type A *C. perfringens* and to a lesser degree other subtypes of *C. perfringens*, causes hydrolysis of membrane phospholipids and thus lysis of RBCs, WBCs, platelets, endothelial cells, and myocytes.[234]

(3) Major laboratory findings in acute, severe cases are anemia, hemoglobinemia, hemoglobinuria, and icterus. Less severe forms are associated with anemia, polychromasia, basophilic stippling, rubricytosis, and leukocytosis.

(4) Similar hemolytic disease has been reported to be caused by a strain of *C. perfringens* type D that produced unusually large amounts of α toxin in addition to ε toxin.[235]

c. *Clostridium perfringens* type A (formerly *C. welchii*) in horses with clostridial abscesses and myositis: Hemolytic anemia has been thought to be immune mediated based on the presence of spherocytes, spheroechinocytes, apparent autoagglutination, or a positive DAT,[114,236] but the spherocytic hemolytic anemia caused by a *C. perfringens* infection in people is reportedly DAT negative and caused by the α toxin phospholipase C lecithinase, which is thought to form spherocytes by hydrolysis of RBC membranes, not via immune mechanisms.[237]

6. EIAV

a. EIAV is a retrovirus that infects cells of the mononuclear phagocytic systems of horses, ponies, donkeys, and mules. The disease is called equine infectious anemia (also known as swamp fever, equine malarial fever, mountain fever, and slow fever).

b. Persistent replication of EIAV in macrophages and a horse's response to the infection create the pathologic states that cause clinical disease.[238] The clinical disease may be acute hemolysis or chronic debilitating disease caused by recurrent episodes.

c. Anemia pathogenesis can involve decreased RBC production and hemolysis. Production decreases because of TNF and other cytokines that inhibit erythroid precursors. Acute hemolytic anemia is due to immune complexes or complement fragments adhered to RBCs, which are then destroyed by macrophages.

d. Major laboratory findings vary. Anemia may appear as an acute intravascular hemolytic anemia, but it more commonly appears as a chronic extravascular hemolytic anemia or a chronic normocytic normochromic anemia. Other findings may include hemoglobinemia (acute), perhaps anisocytosis and macrocytosis, perhaps spherocytosis, thrombocytopenia, neutropenia or neutrophilia, DAT

positivity, and serologic positivity for antibodies to EIAV based on agar gel immunodiffusion (Coggins test) or ELISA results.

7. *Babesia* spp. (named after the Romanian bacteriologist, Victor Babeş)
   a. *Babesia* spp. are protozoa with varied geographical distribution, tick vectors, mammalian hosts, and pathogenicity; infections cause a disease known as babesiosis (babesiasis). *Babesia* spp. are not completely species specific, so organisms typically found in one species may infect another. Babesiosis varies from subclinical disease to severe anemia and death.
   b. These protozoa are typically transmitted via tick saliva; sporozoites invade RBCs and develop into trophozoites and then merozoites which asexually reproduce leading to RBC rupture and invasion of other RBCs. Merozoites may be transmitted horizontally through bodily fluids (bites, blood) or vertically through the placenta.
   c. The intraerythrocytic organisms are referred to as piroplasms (*piro* for the pear shape of some forms), but trophozoites and merozoites are pleomorphic, and may be ring-shaped, tetrad (so-called Maltese cross), ameboid, or anaplasmoid, in addition to piriform. The size of the organisms has been used to group *Babesia* species into large and small subtypes, large organisms being commonly 3–5 µm and rarely up to 6 µm long, while small organisms are typically < 2 µm long.
   d. In dogs, *B. canis*, *B. vogeli*, and *B. rossi* are well-characterized large *Babesia* that have been considered subspecies of *B. canis*; a RBC may contain 1, 2, 4, 8, or rarely more organisms. An unnamed large *Babesia* (often called Coco after the dog it was first described in) infects dogs in the United States, as does *B. vogeli*; *B. rossi* infected an imported dog in Texas.[239] Small *Babesia* that cause disease in dogs are *B. gibsoni*, *B. vulpes* (previously referred to as *Theileria annae*, *B. annae*, or *B. microti*-like piroplasm),[240] and *B. conradae*;[241] infected RBCs typically contain one or two *B. gibsoni* or *B. vulpes* organisms, whereas tetrad forms may occur with *B. conradae*. *B. negevi* piroplasms, described in Israel, are of intermediate size and also form tetrads.[242]
   e. In cats, although several species have been reported, most clinical disease has been associated with *B. felis*, a small piroplasm that is usually present singly or in pairs as rings, piriform bodies, or tetrads. Other species reported to cause disease in cats include *B. leo* and *B. lengau*. Canine subtypes of large *Babesia* have been reported in cats and are thought to cause little clinical disease,[243] but fatal disease attributed to *B. canis* was reported in a young pregnant cat in Spain.[244]
   f. *B. caballi* infects horses with typically two or four organisms per RBC, and *B. bigemina* infects cattle with one or two organisms per RBC. *B. caballi* was detected by PCR in two clinically healthy llamas in a Mexican zoo.
   g. Horizontal transmission of *B. gibsoni* and *B. vulpes* has been associated with dog fighting and American pit bull terrier type breeds, possibly because of transmission of bodily fluids and vertical transmission from dams to pups.[225] Immunocompromised or splenectomized animals may be at increased risk of disease.
   h. Parasitemia: It may be difficult to find organisms in chronic forms of disease. They are more frequently found in capillary blood ear sticks, toenail clips, or in buffy coat preparations. Babesial organisms can induce RBC surface adhesion molecules that bind to endothelial cells, concentrating RBCs in capillary beds and sequestering them away from the animal's mononuclear phagocyte system.
   i. Pathogenesis of anemia appears to involve nonhemolytic and hemolytic mechanisms that may vary with the babesial species.[245] Hemolysis may involve proteases

produced by the invading parasite, an immune reaction to parasitized cells, oxidative damage to RBCs causing eccentrocytosis,[246,247] and/or splenic sequestration associated with splenomegaly.

j. Major laboratory findings
   (1) Chronic form: few to rare organisms in blood, mild anemia, mild lymphocytosis (due to chronic antigenic stimulus), seropositivity to *Babesia* spp., and PCR positivity for *Babesia* spp.
   (2) In acute or subacute forms: many piroplasms in blood, moderate to severe anemia, reticulocytosis and increased polychromasia if not preregenerative (but the response may be less robust than expected for the degree of anemia),[248] macrocytosis, thrombocytopenia, hyperbilirubinemia, bilirubinuria, possibly hemoglobinuria, sometimes spherocytosis, occasionally eccentrocytosis with large babesial organisms, and PCR positivity for *Babesia* spp.
   (3) Not all Babesia spp. detected by molecular techniques are associated with clinical disease. Infections may be multiple.

9. Cytauxzoonosis caused by *Cytauxzoon felis* is discussed in the nonregenerative anemia section. Although destruction of RBCs likely contributes to anemia, marrow responsiveness is typically lacking. However, some cats infected with *Cytauxzoon* spp. that are genetically distinct from *C. felis* have had regenerative anemias.[189]
10. *Theileria* spp.
    a. RBC piroplasms of these protozoa are intracellular and highly pleomorphic, depending on species and stage of parasitemia. Many forms resemble *Cytauxzoon*, but others resemble small babesial piroplasms. An initial extraerythrocytic stage forms schizonts within lymphocytes and monocytes/macrophages after tick transmission; this sets *Theileria* spp. apart from current *Babesia* spp. Iatrogenic transmission through infected blood can occur via merozoites that infect and multiply in RBCs without involvement of WBCs. Vertical transmission from dam to fetus has been reported.
    b. *Theileria equi* (basonym, *Babesia equi*)[249] infects horses and is endemic in many tropical and subtropical areas where it causes equine piroplasmosis. Infected horses may have subclinical disease or pathologic states including intravascular hemolytic anemia, icterus, thrombocytopenia, hepatomegaly, splenomegaly, systemic inflammatory response syndrome, thrombosis, and multiorgan dysfunction. Intravascular hemolysis is thought to occur from merozoite-induced rupture of RBCs; extravascular hemolysis contributes to anemia by removal of infected and noninfected RBCs. A less virulent species, *T. haneyi*, infects horses on multiple continents, including North America.
    c. Over a dozen species infect *Bovidae* worldwide where their vector ticks are found.
       (1) The two most pathogenic species are *T. parva* and *T. annulata*, which cause East Coast fever and tropical theileriosis, respectively; each of these infects and transforms host lymphocytes, inducing them to proliferate, thus promoting survival of the cells and the protozoa.[250] Erythrocytic forms of *T. annulata* cause hemolytic anemia.
       (2) Other species are less pathogenic and nontransforming. Organisms variously identified as *T. buffeli*, *T. orientalis*, or *T. sergenti* in various parts of the world now appear to be a single species, *T. orientalis*, with Buffeli being a benign strain. Some strains (e.g., Ikeda, Chitose) have been associated with outbreaks of clinical disease characterized by fever, anemia, jaundice, lymphadenopathy, diarrhea, stillbirths, and late-term abortions.[250] Major laboratory findings are

piroplasms in RBCs, macrocytosis, polychromasia, basophilic stippling, lymphocytosis, thrombocytopenia, hyperbilirubinemia, and bilirubinuria.[251–253] The many piroplasms present in clinically ill, anemic cattle may be ovoid, racquet shaped, linear, or shaped like signet rings. Piroplasms are rare in subclinical cases.

(3) Theileria-naïve cattle introduced into endemic areas or exposed to infected cattle are especially susceptible. Pathogenesis of the anemia is not established and may include decreased RBC production in addition to extravascular hemolysis from direct damage, immunologic destruction, or oxidative damage.

d. Dogs: PCR testing has detected various *Theileria* species (e.g., *T. annulata*, *T. equi*) in canine blood, and intraerythrocytic piroplasms have been seen microscopically,[254] but clinical significance is unclear. See the *Babesia* section for *T. annae* (*Babesia vulpes*).

e. Although PCR testing can be used to differentiate similar organisms, infections may be mixed, and PCR positivity does not equate with clinical disease.

10. *Rangelia vitalii*

a. Unlike other current Babesia spp., this protozoon of the *Babesiidae* family parasitizes canine endothelial cells, neutrophils, and monocytes in addition to RBCs.[255] However, its reclassification into the *Babesia* genus has been proposed.[256]

b. The major laboratory abnormalities are hemolytic anemia and thrombocytopenia, each of which may involve immune-mediated destruction.[257] Clinical signs of rangeliosis include icterus, pallor, hemorrhage typical of a primary hemostatic defect, and characteristic bilateral pinnal hemorrhage. The disease has been recognized in certain parts of South America.

11. *Trypanosoma* spp.

a. These kinetoplastid protozoa occur as free-living flagellated trypomastigotes in blood plasma and as amastigotes in pseudocysts or macrophages in other tissues. The pathogenicity of the parasitic species varies, and the host specificity for pathogenic species is minimal. PCR testing is needed for reliable speciation.

b. *Trypanosoma theileri* (basonym, *T. americanum*) infects cattle nearly worldwide, being spread through gut contents or feces of tabanid flies, and likely via vertical transmission. The sustained and generally low-level parasitemia is usually of no clinical consequence, but parasitemia (Figure 3.12L) may increase in otherwise compromised cattle, and disease has been attributed to infections in some instances.[258]

c. *Trypanosoma cruzi* infects dogs, cats, and people in South and Central America and in the southern United States of America. It causes Chagas disease. Trypomastigotes are found in blood in the acute stages, but most lesions involve amastigotes in nonhemic tissues (heart, brain, and lymph node). Hemolytic anemia is not a feature of the disorder. Amastigotes of *Trypanosoma* spp. are microscopically similar to those of *Leishmania* spp., both protozoa being kinetoplastids that may be found in macrophages or neutrophils. Amastigotes of *Leishmania* spp. may be seen in blood WBCs,[259] and although amastigotes of *Trypanosoma* spp. are not expected to be found in blood samples, *T. cruzi* amastigotes were found extracellularly in blood smears and buffy coat preparations of blood from a puppy with trypanosomiasis; the blood also contained many trypomastigotes.[260]

d. Major African trypanosomes of veterinary significance are *T. congolense*, *T. vivax*, and *T. brucei*. A mutant form of *T. brucei* – *T. brucei evansi* – has adapted to

mechanical transmission and has therefore spread beyond tse-tse fly territory to North Africa, Asia, and South America. *T. vivax* can also be transmitted mechanically and is present in much of South America. Subspecies of *T. brucei* cause African sleeping sickness in people.

(1) In the acute stages of *T. vivax* infections in cattle, animals may have acute hemolytic anemia, leukopenia, and thrombocytopenia. Nonselective phagocytosis of hemic precursors, RBCs, and platelets has been reported as a prominent finding in marrow, and thus ineffective hematopoiesis associated with a hemophagocytic syndrome contributes to the pancytopenia.[261] Among other mechanisms, trypanosome-derived sialidases may promote cytophagia by desialylating membrane glycoproteins.[262]

(2) *Trypanosoma congolense* infections in cattle and sheep have reportedly caused coating of RBCs and WBCs with trypanosomal antigen-antibody complexes.[263,264]

(3) Horses, donkeys, and dogs can also become anemic from acute and chronic infections by these same three trypanosome species or subspecies.[265] *T. evansi* and *T. brucei* have been reported in cats, but anemia does not appear to be of major significance.

12. Hemolytic anemia occasionally occurs secondary to *Rhodococcus equi* pneumonia in foals; an immune-mediated pathogenesis has been suspected.
13. FeLV: Most anemias associated with FeLV infections are caused by decreased RBC production.[266] One study provided data suggestive of hemolytic anemia, but most data in the study could have represented ineffective erythropoiesis (erythroid hyperplasia) and dyserythropoiesis (macrocytosis and rubricytosis).[267] A FeLV infection may be present in cats that develop unrelated disorders, including immune hemolytic processes, or it may predispose a cat to certain infections (e.g., *Mycoplasma* spp.).

C. Erythrocytic metabolic defects (acquired or inherited)

1. Oxidative damage
   a. Heinz body hemolytic anemia
      (1) Heinz bodies are foci of denatured Hgb. Their formation appears to involve the following sequence of events in RBCs:[129]
         (a) RBCs are exposed to an oxidant that overwhelms the reductive pathways that keep Hgb in a reduced state (Figure 3.5), thus Hgb is converted to metHgb.
         (b) MetHgb undergoes spontaneous conformational changes to form hemichromes (metHgb with unique binding of Fe to nitrogenous bases of globins) or heme-depleted Hgb.
         (c) Hemichromes or heme-depleted Hgb molecules precipitate and aggregate to form Heinz bodies. Oxidation of sulfhydryl groups and formation of disulfide bonds occur during this process. The Heinz bodies are frequently associated with the RBC membrane through hydrophobic bonds.
      (2) Many oxidants or substances metabolized to oxidants are reported to cause Heinz body formation in domestic mammals. They may also cause eccentrocyte formation and/or methemoglobinemia.
         (a) Dogs: acetaminophen (likely the 4-aminophenol metabolite),[268] benzocaine, hydrogen peroxide,[269] *Allium* spp. (e.g., common onions, garlic, and chives containing sodium n-propylthiosulfate, n-propyl

disulfide, and other sulfides),[270,271] vitamin K_1 (phytonadione), vitamin K_3 (menadione), naphthalene, phenylhydrazine, zinc (toys, game pieces, buttons, jewelry, identification tags, tinsel garland, galvanized nuts and bolts, diaper rash cream, zinc supplements, pennies minted in the USA after 1982 and many but not all minted in 1982, and Canadian pennies minted 1977–1999),[272] potentially dipyrone (metamizole),[273] and skunk musk[274] (odor toxicity is usually a greater problem!)

(b) Cats: acetaminophen, benzocaine, methionine, methylene blue (in old urinary antiseptics), onions (*Allium* spp.), phenazopyridine, propofol, propylene glycol,[275] and aniline (metabolites)[276]

(c) Horses: onions, phenothiazine, wilted or dry red maple (*Acer rubrum*) and red maple hybrid leaves or possibly other maple leaves (gallotannins and gallic acid metabolized to pyrogallol by intestinal microbes, or other oxidants),[277] copper,[278] and garlic (*Allium sativum*)[279]

(d) Ruminants: *Brassica* spp. (kale and rape), copper, intravenous hydrogen peroxide (oops! – intended for use in the udder), onions, ryegrass, and possibly sodium chlorate (e.g., herbicide toxicity, though methemoglobinemia is better described);[280] copper deficiency has been associated with Heinz bodies in sheep[170]

(e) Alpacas: wilted red maple leaves (*Acer rubrum*)[281]

(3) Cats are very susceptible to acetaminophen because they lack a glucuronyl transferase that is used by most species to conjugate acetaminophen. Without the conjugation, acetaminophen is converted to reactive metabolites that deplete glutathione concentrations, thus decreasing protection from oxidative injury.

(4) Pathogenesis of the anemia may involve multiple mechanisms:[129]

(a) RBCs containing Heinz bodies are less deformable and are trapped and lysed in the spleen.

(b) Structural damage caused by oxidation of membrane proteins or binding of Heinz body to RBC membrane leads to fragile cells that may lyse within vessels or sinusoids.

(c) Binding of hemichromes to band 3 proteins of the RBC membrane causes a redistribution of band 3 proteins and formation of an antigen that is recognized by autologous antibodies. After antibody binding, the defective RBC is removed by splenic or hepatic macrophages.

(5) Major laboratory features: mild to severe anemia, polychromasia and reticulocytosis, Heinz bodies in blood (NMB or another vital stain may help confirm the presence of Heinz bodies), eccentrocytosis, hyperbilirubinemia and bilirubinuria, hemoglobinemia and hemoglobinuria in severe acute cases, and perhaps methemoglobinemia (dark or chocolate-colored blood)

(6) Feline Heinz bodies

(a) Many clinically healthy, nonanemic cats (or sick cats with or without anemia) have circulating RBCs that contain single small Heinz bodies (diameter ≈ 0.5 μm) that are of little to no clinical significance. Typically, a minority of the RBCs contain Heinz bodies. Larger Heinz bodies or multiple Heinz bodies per cell in an anemic cat should prompt consideration of a Heinz body hemolytic anemia.

(b) Potential reasons for Heinz bodies in healthy cats

(i) Feline RBCs containing Heinz bodies are probably not removed from the circulation as rapidly as in other species because the cat's spleen has a type of open circulation that does not require RBCs to pass through narrow slits from red pulp back to venous outflow; this is thought to decrease the pitting of Heinz bodies.
(ii) Because feline Hgb has more sulfhydryl groups than Hgb of other species, it may form more disulfide bridges and thus more denatured Hgb.
(iii) Feline RBCs may have less reductive capacity.

(c) Cats that ate semimoist food containing propylene glycol had more Heinz bodies, and their RBCs had reduced life spans, but clinically significant anemia was not documented.
(d) Cats with a variety of disorders (e.g., diabetes mellitus, hyperthyroidism, and lymphoma) may have increased percentages of RBCs containing Heinz bodies.
(e) Diagnosis of Heinz body hemolytic anemia in cats requires finding a regenerative anemia, evidence of hemolysis (usually hyperbilirubinemia or bilirubinuria), and demonstration of Heinz bodies in RBCs. Known exposure to an oxidant is helpful.

b. Eccentrocytic hemolytic anemias (acquired or inherited)
(1) Pathogenesis of the anemia probably involves multiple mechanisms. Eccentrocytes are more rigid (less deformable) and may spontaneously rupture in blood (intravascular hemolysis) or be less able to pass through splenic circulation where they are trapped and removed by macrophages (extravascular hemolysis).
(2) Acquired states: Oxidative damage to RBCs may cause formation of eccentrocytes, Heinz bodies, or both (for recognized oxidants, see Hemolytic Disorders, Section C.1.a). Factors that determine which oxidative lesions form are not understood. Oxidants reported to cause Heinz bodies in one animal may cause eccentrocytosis or methemoglobinemia in another. Methemoglobinemia and eccentrocytosis without reported Heinz bodies were attributed to horses ingesting certain species of *Pistacia*.[282] Ingestion of henna containing 2-hydroxy-1,4 naphthoquinone (lawsone) may have caused eccentrocytosis and anemia in a dog.[283]
(3) G6PD (EC 1.1.1.49) deficiency: This X-linked defect is common in people and was found in one American saddlebred colt.[101] The nonsense mutation occurred in the colt's dam[284] and is not a breed problem because none of the colt's male siblings lived to produce progeny. One dog was reported to have RBCs with decreased G6PD activity (44 % of normal), but the dog did not have a clinical disorder related to the defect.[285]
(a) Pathogenesis of anemia: G6PD is the rate-controlling enzyme for the hexose monophosphate shunt from which NADPH is produced (Figure 3.5). NADPH is a potent reducing agent that keeps GSH and, indirectly, other substances in a reduced state. With less G6PD activity, RBCs cannot repair damage caused by spontaneous oxidation, thus they are prone to eccentrocyte and pyknocyte formation and reduced life spans.
(b) Major laboratory findings in horses: persistent macrocytic normochromic anemia (Hct near 20 %), eccentrocytosis and pyknocytosis, macrocytosis, and persistent icterus with primarily increased [Bu]

(c) Confirmatory diagnostic findings: greatly reduced RBC G6PD activity, decreased RBC [GSH] and [NADPH], and increased blood [metHgb]

(4) RBC FAD deficiency: A defective biochemical pathway in the RBCs of a Spanish mustang mare created a FAD deficiency.[102] The FAD deficiency led to deficient activity of Cb_5R and GR, enzymes that contain FAD (Figure 3.5). Hereditary aspects of the defect were not determined. Major laboratory findings were eccentrocytosis, pyknocytosis, Hgb crystals (typically in eccentrocytes), and methemoglobinemia.

2. Defects in ATP generation

a. PK (EC 2.7.1.40) deficiency is an autosomal recessive hereditary disorder in many breeds of dog (basenjis, beagles, cairn terriers, West Highland white terriers, American Eskimo dogs, dachshunds, Chihuahuas, pugs, Labrador retrievers, and others)[286] and many types of cats (Abyssinian, Somali, American shorthair, Bengal, Egyptian mau, laPerm, Maine Coon, Norwegian forest, savannah, Singapura, and others including mixed breed cats).[287,288] The clinical illness typically is seen in dogs in the first few weeks to months of age, but less severe deficiencies can be found in older dogs. Onset in cats is often in the first few years of life, but many cats are asymptomatic, and clinical hemolysis may occur intermittently.[289]

(1) Pathogenesis of anemia: Normal RBCs contain the R-type PK isoenzyme (R-PK, R for RBC) that normally catalyzes the last ATP generation step of anaerobic glycolysis (Figure 3.5). With PK deficiency, young RBCs contain a small amount of R-PK, but the R-PK content decreases as the cell ages. Without R-PK activity, RBCs become ATP deficient, RBC membranes become defective, and hemolysis occurs. RBCs have a greatly decreased life span.

(2) Major laboratory findings in dogs include moderate macrocytic hypochromic anemia, moderate to extreme reticulocytosis (RP = 40–60 %), rubricytosis, typically mild hyperbilirubinemia, hyperferremia, and hyperferritinemia with increased hemosiderin in bone marrow, spleen, and liver. Spheroechinocytes are reported to occur but are uncommon. Pancytopenia may develop in later stages if myelofibrosis and osteosclerosis develop, and Fe overload of the liver may cause cirrhosis.[290] As in people, Fe overload may occur secondary to chronic erythropoiesis and erythroferrone production, thus decreasing hepcidin synthesis and increasing Fe uptake.[291] Osmotic fragility may be mildly increased. Because the defect in glycolysis occurs after the DPG shunt, 2,3-DPG increases, thus shifting the Hgb-O_2 dissociation curve to the right and increasing O_2 delivery to tissue.

(3) Findings in cats vary over time and with severity, usually beginning by 2 yr of age but sometimes not until middle age. Anemia may be absent or mild until unknown triggers precipitate a hemolytic event, and that may not occur until adulthood. Common findings are anemia, aggregate reticulocytosis (which may be marked in the presence or absence of anemia), hyperglobulinemia, and hyperbilirubinemia associated with hemolysis or, rarely, with bilirubin cholelithiasis and cholestasis. Osmotic fragility may be mildly increased, but it is usually WRI.[289,292] Myelofibrosis and osteosclerosis have not been reported.

(4) Confirmatory diagnostic findings include decreased RBC R-PK activity in cats, symptomatic cats having marked reductions, and carriers having approximately 50 % reductions. However, total RBC PK activity may be increased in dogs because of increased heat-labile, M2-type PK isoenzyme

(M2-PK, M for muscle) that persists from developmental erythroid stages. RBC PK activity that is WRI or only mildly increased when marked reticulocytosis is present is suggestive of PK deficiency. PCR testing can confirm PK deficiencies in many dogs and cats, but new mutations may be found. Expected mutations and therefore specific PCR tests vary with the breed for dogs, but a single mutation has been associated with PK deficiency in cats.[287,288,293]

b. PFK (EC 2.7.1.11) deficiency caused by a single nonsense mutation in the gene for muscle-type PFK has been reported as an autosomal recessive disorder in English springer spaniels, American cocker spaniels, whippets, and mixed breed dogs.[294] A different mutation in the gene causes PFK deficiency in Swedish Wachtelhunds.[293,295]
   (1) PFK isoenzymes are tetramers of muscle-type, liver-type, and platelet-type subunits. In adult dogs, PFK in RBCs and myocytes is about 80 % and 100 % muscle-type subunits, respectively, whereas the liver-type subunit is abundant in RBCs of puppies. PFK-deficient puppies have high RBC PFK activity that decreases over about 2–3 mo as fetal RBCs are replaced.
   (2) Although myocytes and RBCs are deficient in PFK, signs of exertional myopathy are often mild or masked and affected dogs (homozygotes) are often presented with exercise-associated hemolytic anemia. They may tire easily and have decreased muscle mass and weakness. Oxidative metabolism may suffice for energy production in myocytes of many dogs, but skeletal and cardiac myopathy was more apparent in PFK-deficient whippets.[294]
   (3) Pathogenesis of anemia: PFK is a rate-controlling enzyme of glycolysis (Figure 3.5). A deficiency in PFK results in less downstream formation of ATP and 2,3-DPG, the major impermeable anion in RBCs of dogs.
      (a) Decreased ATP impairs RBC functions and contributes to premature RBC destruction.
      (b) Decreased [2,3-DPG] results in greater intracellular [Cl^-], greater pH, and enhanced alkaline fragility (normal canine RBCs are generally sensitive to alkalinity). The low [2,3-DPG] also decreases O_2 delivery to tissues such that Epo production is increased. This results in a compensated regenerative state (reticulocytosis with little or no anemia). When hyperventilation occurs because of excitement or exercise, blood pCO_2(a) decreases and alkalemia may lead to a hemolytic crisis.
      (c) Studies of PFK-deficient human RBCs suggest they have a defective Ca^{2+} pump that needs ATP to function, and thus RBCs accumulate Ca^{2+} and become less deformable.[296] However, mechanisms that link the Ca^{2+}-transport defect with the alkaline-induced hemolysis and increased RBC [Cl^-] were not found.
   (4) The major laboratory findings are anemia, hemoglobinemia, and hemoglobinuria after hyperventilation (producing respiratory alkalosis and alkalemia), and hemolytic icterus. When not in an active intravascular hemolytic state, dogs have mild regenerative anemias or reticulocytoses (may be marked) without anemias (compensated hemolysis). Rubricytosis commonly accompanies reticulocytosis. Hyperkalemia may be present because of in vitro leakage of K^+ from reticulocytes.[295] Osmotic fragility is not increased, but alkaline osmotic fragility is.[294]

(5) Confirmatory findings: PFK-deficient RBCs (after about 3 mo of age), decreased RBC [2,3-DPG], increased Hgb affinity for oxygen, and increased RBC [Cl^-]. PCR testing can confirm PFK deficiency and carrier states; Wachtelhunds have a different mutation and require a different PCR test than other dogs reported to date.[293]

c. Hypophosphatemic hemolysis

(1) Postparturient hemoglobinuria in cows, typically 2–8 wk after calving[297]

(a) Pathogenesis of anemia: The pathogenesis is unclear and may be varied and multifactorial, but hypophosphatemia has been implicated as a cause or contributor.[125,298] Defective mobilization of phosphorus from bone and increased phosphorus loss via milk may contribute to hypophosphatemia and decreased [phosphate] within RBCs. This may limit ATP production and promote hemolysis, but other factors seem to be involved. When hypophosphatemia was induced by a low phosphorus diet for 4 wk before and 4 wk after parturition, some cows developed overt hemoglobinuric anemia and others had milder hemolytic anemias.[125]

(b) Major laboratory findings: hypophosphatemia in some cows, hemoglobinemia, hemoglobinuria, and moderate to marked anemia; Heinz bodies and spherocytes have also been reported[125,299]

(2) There are sporadic reports of concurrent hypophosphatemia and severe hemolysis in horses, dogs, and cats (e.g., following insulin therapy).[300,301] Severe hypophosphatemia may decrease RBC ATP production and thus produce unstable RBC membranes and hemolysis. In dogs, a low [2,3-DPG] may also play a role by making cells more susceptible to alkaline-associated hemolysis.

(3) Bilirubin interference can cause artifactual hypophosphatemia with some assay methods. This must be excluded before attributing a hemolytic anemia to hypophosphatemia in an icteric patient.

(4) Hyperinsulinism (primary or secondary to hyperglycemia) can cause hypophosphatemia by promoting movement of glucose and phosphate into cells other than RBCs (transport into RBCs is not insulin dependent). Persistent hypophosphatemia may deplete RBC phosphate, decrease ATP production, and cause hemolysis.

d. L-Sorbose intoxication:[302] Most dogs (those with low-K^+ RBCs) develop hemolytic anemia after ingestion of L-sorbose, a sugar substitute. Dogs with high-K^+ RBCs (e.g., large minorities of Japanese shiba inu and Akita dogs) and human, cattle, and equine RBCs are resistant to the effects of L-sorbose. Evidence indicates that the hemolysis is due to ATP depletion that results from the strong inhibition of hexokinase by sorbose-1-phosphate in dogs with low-K^+ RBCs. Hexokinase converts glucose to glucose-6-phosphate in the initial step of glycolysis (Figure 3.5). Sorbose-1-phosphate is also formed within high-K^+ RBCs but has less effect on their hexokinase.[302] Although the traits are not linked genetically, many dogs of breeds with high-K^+ RBCs also express GLUT1 on RBCs;[15] it is not clear if expression of this sugar (and L-sorbose) transporter impacts the effects of L-sorbose.

3. Defects in heme synthesis that cause porphyria

a. Porphyrias are a group of hereditary and acquired disorders in which porphyrins accumulate in cells and body fluids because of deficient enzyme activity in the heme synthetic pathway (Figure 3.2). Collectively, porphobilinogen through

protoporphyrin IX are called porphyrins. Some authors define porphyria as an inherited disorder and porphyrinuria as an acquired disorder with excess excretion of mostly coproporphyrin (e.g., lead poisoning).[303]

(1) Some forms of porphyria produce a hemolytic anemia, but others do not. The accumulation of certain porphyrins in RBCs reduces RBC life span and thus causes hemolytic anemias. The mechanism of RBC destruction is not firmly established but may relate to porphyrin-induced damage of membrane lipids or photolysis when RBCs are near the skin surface.[303] When certain porphyrins absorb ultraviolet light, excitation of the molecule leads to oxidative damage to cells. Another common manifestation of oxidant damage is the dermatitis of photosensitivity, which affects nonpigmented or less-pigmented skin.

(2) Congenital (hereditary) porphyrias can be classified as either erythropoietic or hepatic depending on the major cell type expressing the enzymatic defect in heme synthesis.[303]

(3) The acquired (secondary) hepatic porphyrias are more common. Cattle and horses may develop porphyria and the resulting photosensitization when severe hepatobiliary disease decreases biliary excretion of phylloerythrin, a porphyrin derived from the breakdown of chlorophyll.

b. Congenital erythropoietic porphyria

(1) Bovine congenital erythropoietic porphyria: The homozygous calves with this autosomal recessive disorder have reddish brown discoloration of teeth and bones, photosensitivity, and hemolytic anemia of varying severity. The degree of regeneration corresponds to the degree of anemia. The disorder has been found in many breeds of cattle including Holsteins, shorthorn, longhorn, and Jamaican cattle. Clinical severity relates mostly to the degree of deficiency in uroporphyrinogen III synthase, an enzyme that catalyzes reaction 4 in Figure 3.2. Deficiency causes formation of porphyrinogens which cannot be converted to heme and are oxidized to uroporphyrin I and coproporphyrin I. These porphyrins accumulate in erythroid precursors, plasma, growing teeth and bones, feces, and urine. Excess incorporation into RBCs induces hemolysis. Porphyrin-rich urine, teeth, bones, or erythroid cells may fluoresce under ultraviolet light.

(2) Feline erythropoietic porphyria: Erythropoietic porphyria was diagnosed in a 10-yr-old castrated male domestic shorthair cat with dark red urine, brownish teeth that fluoresced red under ultraviolet light, increased uroporphyrin I in urine and plasma, decreased uroporphyrinogen III synthase activity in RBCs, and two mutations in the gene for this enzyme.[304] The cat was not anemic, but had mild rubricytosis, polychromasia, and basophilic stippling. The disorder has been suspected in other cats, some with hemolytic anemia and renal disease, but specific enzyme defects were not established in these cats.[305,306] Cats with acute intermittent porphyria caused by a deficiency of hydroxymethylbilane synthase (porphobilinogen deaminase, reaction 3 in Figure 3.2) can have similar clinical findings and mild changes in the erythron.[306] Inclusions resembling irregular Howell–Jolly bodies have been reported in polychromatophils, in fewer mature RBCs, and in few neutrophils of an affected cat.[307]

c. Other hereditary erythropoietic porphyrias including erythropoietic protoporphyria (ferrochelatase defect) have been diagnosed in domestic mammals, but anemia is mild or not an expected feature.[83,303]

d. Lead poisoning creates an acquired porphyria because lead inhibits enzymes in the heme synthetic pathway. Anemia may be present in plumbism, but it is not considered a hemolytic anemia caused by the accumulation of porphyrins.

D. RBC fragmentation in blood
   1. RBC damage causing formation of schistocytes and keratocytes is thought to be due to intravascular trauma generated by relatively rigid structures (fibrin) or by rheologic forces (Table 3.12 for disorders).
   2. Pathogeneses of anemia: Because the RBC trauma is a consequence of other pathologic states, processes that cause the anemia may be multifaceted.
      a. RBC trauma either directly causes lysis or creates poikilocytes that have a shortened life span. If acanthocytes are present from underlying disease that creates them, these forces may contribute to the accelerated fragmentation of acanthocytes (budding fragmentation).
      b. Primary diseases are frequently infectious or noninfectious inflammatory disorders, and thus the inflammatory state may be producing anemia (see Nonregenerative Anemias, Section II.A.1).
   3. Major laboratory findings may include mild to moderate anemia with no reticulocytosis or moderate reticulocytosis and polychromasia, schizocytosis and keratocytosis that can be marked, and thrombocytopenia. There may be other evidence of consumptive coagulation or a consumptive coagulopathy. There may be evidence of renal failure with hemolytic uremic syndrome.

E. Hemolytic disorders of other or unknown pathogeneses
   1. Idiopathic nonspherocytic hemolytic disorders with increased osmotic fragility
      a. Hereditary nonspherocytic hemolytic anemia in beagles: Affected beagles had mild chronic anemia (Hct values of 30–39 %), persistent reticulocytosis, splenomegaly, erythroid hyperplasia in marrow samples, shortened RBC life spans, and increased RBC osmotic fragility.[308,309] Studies suggest that the defect was an autosomal recessive trait, but the specific defect has not been established.
      b. Idiopathic hemolytic anemia in cats, including Abyssinian and Somali cats:[310,311] A total of 18 Abyssinian (13) and Somali (5) cats, male and female, had macrocytic, regenerative anemias (Hct values of 5–25 %), splenomegaly, and increased RBC osmotic fragility that appeared to be an autosomal recessive trait. The specific defect has not been established. Similar findings of increased osmotic fragility, splenomegaly, and moderate to marked macrocytic hypochromic anemia with various degrees of regeneration have been reported in other breeds and in domestic shorthair cats.[311] In vitro hemolysis may develop within hours and become marked within a day, or overnight in some cases. Other findings may include lymphocytosis, hyperglobulinemia, rouleaux, and Fe overload.
   2. Hypoosmolar hemolysis
      a. Rapid infusion of hypotonic fluid intravenously (such as sterile H_2O) or the ingestion of large quantities of water by calves (water intoxication) can cause rapid intravascular hemolysis.[312]
      b. Pathogenesis of the anemia: Infusion of a hypotonic solution or absorption of large quantities of H_2O creates hypoosmolar plasma, either in vessels adjacent to the alimentary tract where H_2O is absorbed, or systemically. Rapid movement of H_2O into RBCs via osmosis causes RBC swelling and lysis. Prior water restriction may be a predisposing factor, as may an increased osmotic fragility of calf RBCs.[312,313]

c. The major laboratory findings are anemia, hemoglobinemia, hemoglobinuria, hyponatremia, and hypochloremia, the severities of which depend on the severity of the hypoosmolar state.

3. Envenomation: Venoms from some animals (e.g., certain snakes, spiders, and insects) cause hemolysis and may cause hemolytic anemias.[113,126]
   a. Mechanisms of hemolysis include complement activation with subsequent RBC destruction (e.g., cobra venom factor) and direct hemolysis from hemolysins. Hemolysins include phospholipase A_2 in rattlesnake and honey-bee venoms, and melittin in honey-bee venom.[111,113]
   b. Echinocytosis, spherocytosis, echinospherocytosis, ghost cells, or so-called erythroid loops may be present, depending on the venom, the dose, and the time from envenomation.[111,113,127] Echinocytosis occurs from membrane changes that are often attributed to phospholipase effects. Spherocytosis may relate to altered membranes, altered cytoskeletal proteins, or possibly antibody-mediated changes. Ghost cells may occur with membrane pore formation from complement activation or without complement involvement (e.g., melittin). The erythroid loops reported in dogs with hemolytic anemia after envenomation by vipers (*Vipera aspis*) were ring-shaped membrane-like structures of unknown pathogenesis and composition.
   c. Elapid snake envenomation or its treatment (including use of antivenom) were suspected to have precipitated IMHA in dogs.[314]
4. Histiocytic neoplasia: When anemia accompanies hemophagocytic histiocytic sarcoma (malignant histiocytosis), extravascular hemolysis by neoplastic cells is one contributing factor to an animal's anemia.
5. Hemophagocytic syndrome: Nonselective phagocytosis of hemic cells by nonneoplastic, cytokine-stimulated macrophages may contribute to anemia and other cytopenias in a variety of inflammatory or neoplastic disorders.
6. Heparin-induced hemolysis
   a. Unfractionated heparin anticoagulant therapy in horses can cause RBC agglutination, mild to moderate anemia, and increased biliary bilirubin excretion that reflects increased Hgb degradation.
      (1) Pathogenesis of the decreased Hct appears to involve hemolysis and in vivo RBC agglutination that promotes entrapment and phagocytosis.[315] The effects of heparin can be inhibited and reversed in vitro by trypsin administration.[316]
      (2) Artifactual anemia is also present when samples are assessed by hematology analyzers; this accounted for about half the decrease in calculated Hct for samples from heparin-treated horses.[316] RBC agglutination causes an erroneously low [RBC] and erroneously high MCV because a group of agglutinated cells is considered one large cell. Therefore, the falsely decreased measured [RBC] can produce a falsely low calculated Hct. Agglutinates may be excluded from counting and from inclusion in the MCV calculation, thus not affecting MCV but still causing a falsely decreased [RBC] and therefore a falsely decreased calculated Hct and falsely increased MCHC and MCH.
   b. Major laboratory findings: In experimental studies, spun Hct (microhematocrit) decreased from near 30 % to near 20 % within 6–8 h after heparin treatment and then returned to baseline percentages by day 5. Bilirubin concentrations increased from near 0.6 mg/dL to 1.4 mg/dL within 24 h of heparin treatment.[315]
7. Hemolytic syndrome in horses with hepatic disease:[317,318] Horses with hepatic or hepatobiliary disease have developed intravascular hemolytic anemia with

hemoglobinuria that did not appear attributable to other disease. Some of the horses were considered to have pyrrolizidine alkaloid toxicity. The cause of the hemolytic state has not been determined; bile acids have been considered a potential cause.

ERYTHROCYTOSIS AND POLYCYTHEMIA

I. Terms and concepts
   A. Erythrocytosis
      1. *Erythrocytosis* is an increased [RBC] in blood, just as a leukocytosis is an increased [WBC] in blood. It is typically accompanied by an increased Hct and increased blood [Hgb], although abnormalities in RBC volumes and Hgb content can affect alignment of these values. For example, Hct and [Hgb] may be mildly increased without erythrocytosis if the mean RBC volume and mean RBC Hgb content (MCH) are increased.
      2. In human medicine, the term has commonly been applied to an increase in whole-body RBC mass, whether or not the [RBC] or Hct is increased. That definition is not used in this textbook.
   B. Polycythemia
      1. *Polycythemia vera* (polycythemia rubra vera) is a myeloproliferative neoplasm characterized by a clonal proliferation of hematopoietic precursors that produce erythrocytosis and often leukocytosis and thrombocytosis. In this context, polycythemia means "many" (poly-) "cells" (cyth-) in the "blood" (-emia).
      2. *Polycythemia* is commonly used synonymously with erythrocytosis. Because that use can lead to confusion and the term is less descriptive for an increase in [RBC], erythrocytosis and polycythemia are not considered synonyms in this textbook. Polycythemia is used only in the context of the neoplastic state of polycythemia vera.
   C. Erythrocytosis versus increased whole-body RBC mass
      1. Because [RBC] is determined by both the number of RBCs in circulation (numerator) and the circulating fluid volume (denominator), an increase in the former or a decrease in the latter may cause erythrocytosis. Whole-body RBC mass (of the total number of RBCs in the body) may or may not be increased. Hct and [RBC] are not measures of mass, as indicated by their units of measure. RBC mass is difficult to measure.
      2. *Hemoconcentration*, commonly caused by dehydration, is an increased concentration of blood components (including RBCs) because of decreased plasma volume. It causes erythrocytosis without an increase in the circulating or whole-body RBC mass. This is often referred to as a relative erythrocytosis (polycythemia) and has also been called spurious erythrocytosis, but it is absolutely an erythrocytosis as defined by an increased [RBC].
      3. Erythrocytosis caused by erythroid hyperplasia or neoplasia reflects an increased whole-body RBC mass. This is often referred to as absolute erythrocytosis (absolute polycythemia). Mild increases in whole-body RBC mass may be present without erythrocytosis when there is concurrent plasma volume expansion. In human medicine, this state has been recognized by measuring the dilution of injected radiolabeled RBCs and albumin to determine whole-body RBC mass and plasma volume, respectively.
      4. Erythrocytosis also may be caused by splenic contraction and redistribution of RBCs from the spleen to blood. Like the erythrocytosis of hemoconcentration, there is not an increase in whole-body RBC mass. This has been referred to as a relative, apparent, or spurious erythrocytosis (polycythemia). As defined by an increased [RBC], it is absolutely an erythrocytosis; it is not spurious or just apparent.

D. Extreme erythrocytosis may cause sludging of blood and thus impaired blood flow and poor oxygenation of tissues. Related clinical signs may include purplish mucous membranes, congested retinal blood vessels, seizures, mentation changes, and weakness. Blood [glucose] may be falsely decreased (see Chapter 14).

II. Erythrocytotic disorders and conditions (Table 3.15)

A. Before pursuing causes of erythrocytosis, breed differences should be considered. Some breeds have greater RBC concentrations than others, so applicable, valid, breed-specific RIs should be considered when available.

1. Thoroughbreds, standardbreds, and quarter horses have greater Hcts than draft horses.
2. Greyhounds, Afghan hounds, salukis, and whippets have greater Hcts than most other dogs. The Hcts of the former average in the high 50s to low 60s,[133] whereas most dogs have mean Hcts in the mid to high 40s in health. The mean Hct for dachshunds may be in the low 50s.

B. Hemoconcentration

1. Dehydration is the most common cause of erythrocytosis in mammals; dehydration creates a mild to moderate erythrocytosis of no direct pathologic significance. Erythrocytosis is not due to an increased mass of erythroid cells in the body.

Table 3.15. Disorders and conditions that cause erythrocytosis

Transient erythrocytosis
Hemoconcentration
Dehydration*
Endotoxic shock
Splenic contraction*
Persistent erythrocytosis
Secondary appropriate erythrocytotic disorders
Right-to-left shunts, congenital or acquired
Chronic pulmonary disease (typically mild if present)
Hyperthyroidism (typically mild if present)
Persistent methemoglobinemia
(Hemoglobinopathies)[a]
Secondary inappropriate erythrocytotic disorders
Renal neoplasms, cysts, or other disorders
Other neoplasms (e.g., hepatoma, leiomyosarcoma)
(Hypoxia sensor defect)[a]
Primary erythrocytotic disorders
Polycythemia vera (polycythemia rubra vera)
(Epo receptor defect)[a]
Idiopathic erythrocytosis

* A relatively common disorder, condition, or mechanism

[a] Reported in people but not yet in domestic mammals

Note: Animals that live at higher altitudes and perhaps those that have been physically trained (e.g., racing animals) have an increased need for Hgb to transport O_2 to tissues and may produce more RBCs. In addition, certain breeds of dogs and horses have greater Hct values than other animals of the same species (see list in Erythrocytosis and Polycythemia, Section II.A).

a. Pathogenesis of the erythrocytosis of hemoconcentration is shown in Figure 3.23.
b. Laboratory findings that support a conclusion that erythrocytosis is caused by dehydration are (i) hyperproteinemia, possibly with concurrent hyperalbuminemia, (ii) hypernatremia accompanied by hyperchloremia if dehydration is hypertonic (net loss of hypotonic fluid), and (iii) correction with fluid administration.

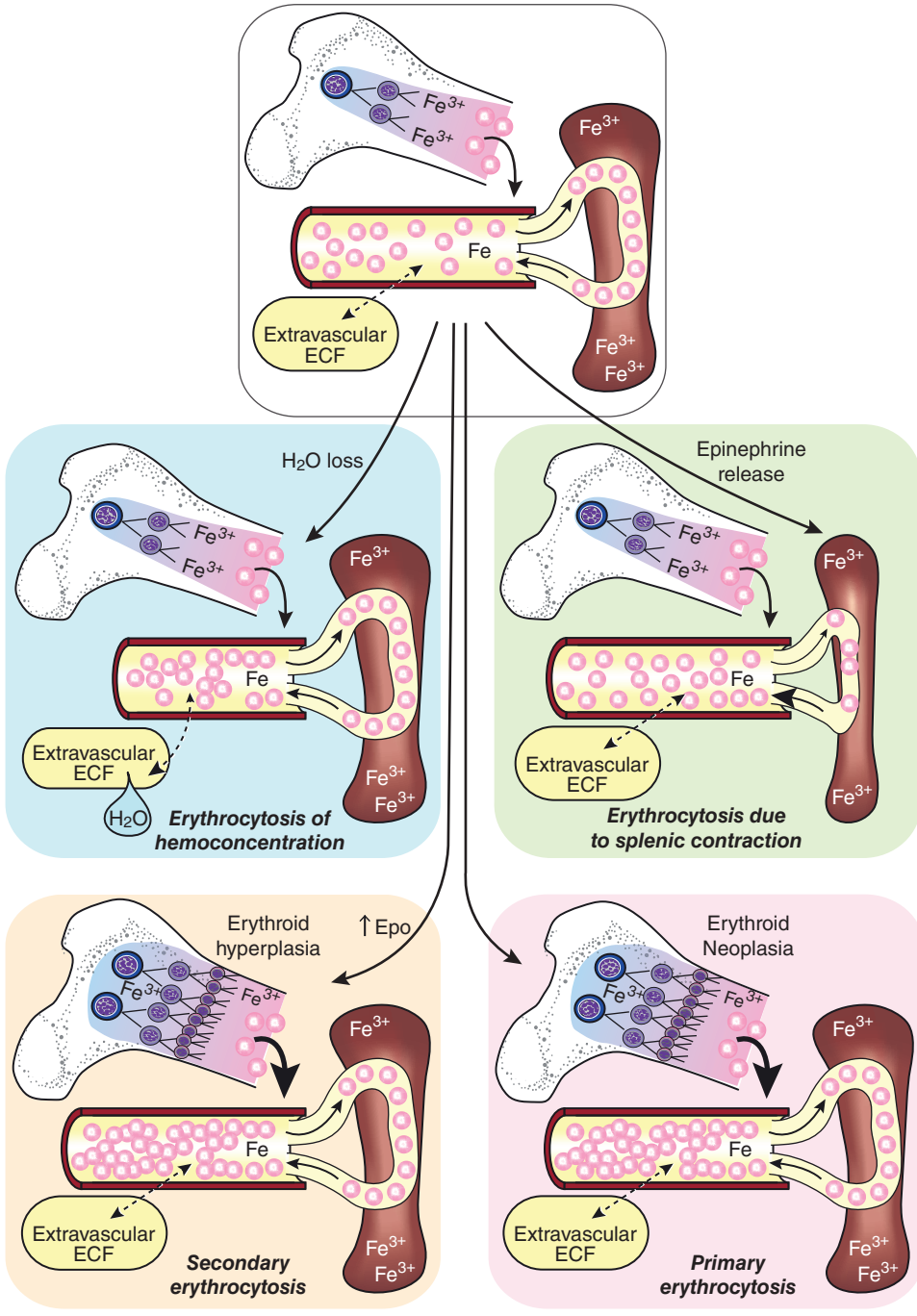


Figure 3.23. Pathogeneses of the erythrocytoses.

2. Endotoxic shock, occurring in some animals with endotoxemia, may cause hemoconcentration because of a shift of H_2O from intravascular to extravascular space (not loss from the body, so not dehydration). Erythrocytosis is usually mild to moderate and of no direct pathologic significance.
   a. Pathogenesis of the erythrocytosis: Endotoxins bind to endothelial cells and cause endothelial barrier dysfunction with intercellular gaps such that blood vessels become more permeable to proteins. Increased extravascular proteins reduce the oncotic pressure gradient, which promotes the shift of plasma H_2O from intravascular to extravascular space, thus causing a decrease in plasma volume and an erythrocytosis.
   b. Laboratory findings that support endotoxic shock include an inflammatory leukogram (either neutropenia or neutrophilia) and thrombocytopenia. Erythrocytosis resolves with successful therapy.

C. Splenic contraction (physiologic erythrocytosis) (Figure 3.23)
1. This state is called physiologic erythrocytosis because it results from physiologic responses to excitement, fright, and exercise that cause epinephrine release from adrenal medullae. After epinephrine injections in cats, Hct values were about 25 % greater than pre-injection.[21] Five minutes after phenylephrine injections in horses, Hct values had increased by about 30 %.[319]
2. Erythrocytosis is not due to an increased mass of erythroid cells or RBCs in the body, but the RBC number and concentration are increased in the circulating blood. Erythrocytosis is mild to moderate and of no direct pathologic significance.
3. Laboratory findings include a transient erythrocytosis, the [RBC] returning to WRI after the stimulus is gone, and possibly a physiologic leukocytosis with mature neutrophilia and lymphocytosis.

D. Secondary appropriate erythrocytosis (sustained hypoxic erythrocytosis) (Figure 3.23)
1. This state is called secondary because erythroid cell proliferation is stimulated by Epo rather than being autonomous. It is called *appropriate* because Epo is increased by renal (usually systemic) hypoxia rather than being autonomously produced. Erythrocytosis and erythroid hyperplasia are due to increased RBC production stimulated by Epo.

Figure 3.23. *continued*

- Erythrocytosis of hemoconcentration (*blue background*): Dehydration (decreased total body water) results in a decreased ECF volume and thus a decreased plasma volume. With a decreased plasma volume but no change in the number of blood RBCs, the [RBC] is increased. Loss of plasma H_2O because of increased vascular permeability in endotoxic shock also causes erythrocytosis via hemoconcentration.
- Erythrocytosis due to splenic contraction (*green background*): The fight-or-flight response or exercise causes release of epinephrine which causes contraction of splenic smooth muscle and thus release of splenic blood into circulation. Splenic blood with a high Hct (70–80 %) is mixed with peripheral blood (Hct = 40–50 %) to cause this physiologic erythrocytosis. Epinephrine administration has a similar effect.
- Secondary erythrocytosis (*tan background*): Increased Epo activity stimulates erythropoiesis to cause erythroid hyperplasia and erythrocytosis associated with an increase in total body RBC mass. Increased Epo production may be appropriate (if stimulated by tissue hypoxia) or inappropriate (if not stimulated by tissue hypoxia). Several disease states can cause secondary erythrocytosis (see Erythrocytosis and Polycythemia, Sections II.D and E).
- Primary erythrocytosis (*pink background*): There is a proliferation of erythroid cells in the absence of increased Epo production that causes an erythrocytosis and increases total body RBC mass. This may be neoplastic or nonneoplastic. Nonneoplastic primary erythrocytosis caused by defective Epo receptors occurs in people. If erythrocytosis is concurrent with a neoplastic proliferation of WBCs and megakaryocytes, then polycythemia vera is present.

2. Disorders that cause hypoxic erythrocytosis
   a. Cardiac disease that leads to persistent poor perfusion of the lungs, i.e., congenital right to left shunts, including atrial or ventricular septal defects or the tetralogy of Fallot
   b. Pulmonary disorders that cause persistent poor oxygenation of blood, although erythrocytosis is uncommon and generally mild when present[320]
   c. Hyperthyroidism that increases the metabolic rate and thus increases the need for O_2 in tissues; in one study, about 45 % of 131 hyperthyroid cats had erythrocytosis (Hcts up to 57 %),[321] but not all studies have identified erythrocytosis in hyperthyroid cats
   d. Persistent methemoglobinemia (e.g., congenital Cb_5R deficiency)
   e. Although not reported in domestic mammals, a defective Hgb molecule (hemoglobinopathy) that has decreased ability to transport or release O_2 can cause erythrocytosis. Acquired disorders (such as cyanide poisoning, carbon monoxide poisoning, and nitrate poisoning) are typically acute and thus of insufficient duration to cause erythrocytosis.
3. Pathogenesis: Hypoxemia, increased tissue O_2 consumption (hyperthyroidism), or potentially decreased O_2 delivery causes sustained tissue (renal) hypoxia, which leads to increased Epo production, increased erythropoiesis via erythroid hyperplasia, and, with time, erythrocytosis.
4. Clinical data that would support secondary appropriate erythrocytosis include decreased pO_2(a) (< 80 mmHg, strongly if < 70 mmHg[322] or < 60 mmHg[323]), decreased oxygen saturation (< 90–95 %), and a history or evidence of a right to left shunt, chronic pulmonary disease, or hyperthyroidism. Blood gas guidelines should be based on altitude-appropriate reference values. Demonstration of a hemoglobinopathy could also be supportive.
5. Physiologic processes may cause or mimic secondary appropriate erythrocytosis.
   a. Mammals that move to high enough altitudes may develop tissue hypoxia. The hypoxia stimulates Epo production and erythropoiesis, resulting in erythroid hyperplasia and erythrocytosis that resolve the tissue hypoxia.[324] With noted exceptions,[325] such mammals are expected to have greater Hcts than mammals living at sea level, but without tissue hypoxia or pathologic consequence at most elevations. Rather than erythrocytosis, this can be considered to reflect health in a different environment for which higher RIs are appropriate. However, there can be pathologic consequences of increased RBC mass at high altitudes.
   b. Prolonged exercise training (conditioning) in horses has inconsistently yielded mild increases in Hct.

E. Secondary inappropriate erythrocytosis (Figure 3.23)
1. This rare state is called *secondary* because erythroid proliferation is secondary to stimulation by Epo rather than being autonomous. It is called *inappropriate* because the increased Epo production is autonomous rather than being caused by hypoxia. Erythrocytosis and erythroid hyperplasia can be mild to marked.
2. Pathologic causes
   a. Inappropriate Epo production by renal tissue because of renal cysts, renal neoplasms (carcinomas, sarcomas, lymphomas), and, rarely, other renal disease including pyelonephritis; renal neoplasms may produce Epo (or Epo-like substance) or promote Epo production by nonneoplastic tissue

b. Inappropriate Epo production by other benign or malignant neoplasms (e.g., hepatoma, hepatoblastoma, schwannoma, leiomyosarcoma, hemangiosarcoma); some of these (e.g., hepatoblastoma in horses, nephroblastomas in dogs) may occur in young or older animals

c. Although not reported in domestic mammals, congenital secondary inappropriate erythrocytosis may occur from mutations affecting renal O_2 sensing or response through the hypoxia inducible factor (HIF) pathway resulting in HIF-2α and Epo production without hypoxia.

3. Clinical data supporting that a persistent erythrocytosis is secondary and inappropriate are exclusion of transient and secondary appropriate erythrocytoses and detection of a known associated pathologic disorder (e.g., a renal neoplasm). If a validated assay for Epo is available, an increased [Epo] is supportive of secondary erythrocytosis. However, [Epo] may not be increased if erythrocytosis has resolved tissue hypoxia and there is no longer a strong stimulus for Epo production at the time of testing. Also, [Epo] can be WRI or even increased with primary erythrocytosis if marked erythrocytosis causes poor tissue perfusion and renal hypoxia (test after phlebotomy).

F. Primary erythrocytosis (Figure 3.23)

1. This state is called *primary* because erythroid cell proliferation is autonomous rather than secondary to Epo. It may be neoplastic and acquired or nonneoplastic and congenital. Disorders in this erythrocytotic group can cause mild to marked erythrocytosis.
2. Disorders
   a. Polycythemia vera is a myeloproliferative neoplasm of erythroid, myeloid, and megakaryocytic cell lines. Primary erythrocytosis may be the presenting abnormality. In people, it is commonly associated with a mutation in the Janus kinase 2 gene (*JAK2*) that causes increased activity of JAK2 kinase, an enzyme involved in signal transduction from the Epo receptor. This promotes cell proliferation in the absence of appropriate cytokine stimulation. Such a mutation has supported the diagnosis of polycythemia vera in a dog with erythrocytosis.[326]
   b. In people, mutations causing truncation of the Epo receptor can also lead to sustained Epo stimulation and congenital nonneoplastic primary erythrocytosis that may be clinically silent and not recognized until adulthood. This isolated erythrocytosis disorder has not been identified in domestic mammals but should be considered as a potential cause.
   c. In a series of 18 cats with primary erythrocytosis based on exclusion of other causes, the trilineage involvement expected with the most common type of *JAK* mutation in people was not present.[327] Different *JAK* mutations or gain-of-function Epo receptor gene mutations may have been causative.
3. Support for these disorders includes the absence of other causes of erythrocytosis, and, in the case of polycythemia vera, bone marrow with too many erythroid cell, myeloid cell, and megakaryocyte precursors (may be difficult to determine). Identification of a typical mutation associated with polycythemia vera (*JAK2* mutation) or the Epo receptor would also be supportive. Theoretically, blood [Epo] would not be increased with primary erythrocytosis. However, blood [Epo] may be increased because of hypoxia induced by a concurrent disorder or by marked erythrocytosis and resultant sluggish flow and poor renal perfusion.

G. Idiopathic erythrocytosis is the classification for patients whose erythrocytosis cannot be classified.

RETICULOCYTOSIS WITHOUT ANEMIA

I. General Concepts
   A. When automated reticulocyte assessment became a routine component of veterinary hematology analyzers, the occurrence of reticulocytosis without anemia became more apparent. Prior to that, evaluation of reticulocytosis was mostly limited to anemic patients.
   B. Values indicative of reticulocytosis, defined as a RC (or CRP) greater than the upper reference limit, vary. This is partly because of variations in methods (analyzer, dye, gating thresholds) and lack of a gold standard. The microscopic method is imprecise, making comparisons and alignment with automated analyzers problematic.
   C. Despite the method, typical RIs are generated to include about 95 % of healthy individuals, so one would expect about 2.5 % of healthy (therefore nonanemic) individuals to have a reticulocytosis (usually mild) with valid RIs.
   D. In studies of various canine and feline populations, including sick animals, reticulocytosis without anemia was identified in about 3–7 % of dogs or cats. In one study that omitted animals with erythrocytosis, it was present in 3.1 % of cats and 4.4 % of dogs overall, and in 1.8 % and 1.5 % of healthy cats and dogs, respectively.[138] It was therefore more common in illness than in health, occurring in a wide variety of disorders including blood loss, cardiac disease, pulmonary disease, gastrointestinal disease, inflammatory disease, and cancer. The low percentages of reticulocytosis in health (< 2.5 %) suggest that the upper reference limit may have been high, so the prevalence in sick dogs and cats might be somewhat greater than reported. Manufacturer reference values were used to identify anemia and reticulocytosis.

II. Potential causes of reticulocytosis without anemia (may be multifactorial)
   A. Pseudoreticulocytosis (see Anemia, Section II.A.3)
   B. Regenerative anemia, but with the anemia masked by hemoconcentration
   C. Reticulocyte shifting to blood
      1. Catecholamine-induced splenic contraction may shift a disproportionate number of reticulocytes to blood and cause transient mild reticulocytosis. Reticulocytes may be relatively increased in splenic blood because of delayed transit as they undergo final maturation.
      2. Exercise may shift reticulocytes to blood from spleen or, perhaps, bone marrow.[328]
   D. Renal hypoxia without anemia, causing increased Epo production and erythropoiesis
      1. Cardiovascular disease (may even cause secondary appropriate erythrocytosis)
      2. Upper or lower respiratory disease such as cardiogenic pulmonary edema associated with myxomatous mitral valve disease[329] and perhaps even dogs with respiratory difficulty from being brachycephalic
      3. Abnormal Hgb (not identified yet in domestic mammals) or decreased oxygen delivery from Hgb, e.g., with decreased RBC [2,3-DPG] in dogs that are heterozygous carriers of PFK deficiency
   E. Compensated or partially compensated blood loss or hemolysis
      1. Mild blood loss assessed at the right time may yield an appropriate reticulocytosis without anemia, either because the Hct never dropped below the lower reference limit or because the Hct has corrected. This may be seen transiently 1–2 wk after blood donation or with hemorrhage.
      2. Hemolysis may be ongoing and compensated enough that reticulocytosis is present without anemia, e.g., with hereditary or acquired metabolic hemolytic disorders.

F. Hormonal stimulation other than by Epo may occur in disorders with increased production of cortisol, androgens, or thyroxine.
G. Certain types of dyserythropoiesis[65]
H. Primary and secondary erythrocytoses

OTHER RBC DISORDERS

I. Methemoglobinemia
  A. *Methemoglobinemia* is the condition in which metHgb (Fe^{2+} oxidized to Fe^{3+}) accumulates in RBCs. It may be hereditary or acquired.
    1. Hereditary defects in RBC reductive pathways
      a. Cb_5R deficiency in dogs (several breeds) and cats
      b. Associated with RBC FAD deficiency in horses[102]
      c. GR deficiency in a horse[330]
    2. Acquired
      a. Methemoglobinemia without Heinz body or eccentrocyte formation
        (1) Nitrate and nitrite toxicity: In ruminants and camelids, ingestion of feed with high nitrate content increases rumen formation and absorption of nitrites. Nitrite (NO^{2-}) binds to oxyhemoglobin and results in release of O_2 and chain reactions that form metHgb, hydrogen peroxide, nitrous dioxide radical, and more nitrite. Nitrite toxicity has occurred in dogs and cats that ate excessive sodium nitrite used as a preservative. Anemia is not expected.
        (2) Hydroxyurea (hydroxycarbamide): Therapeutic doses have caused methemoglobinemia in dogs[331] and cats;[327] anemia is not expected.
        (3) Sulfates in drinking water have been reported to cause mild methemoglobinemia and sulfhemoglobinemia in cattle.[332]
        (4) Sepsis may rarely cause mild methemoglobinemia, apparent only by metHgb assessment.[333]
      b. Methemoglobinemia with Heinz bodies or eccentrocytes: A variety of toxicants are oxidants that overwhelm the reductive capacity of RBC pathways and thus can lead to conversion of Hgb to metHgb. Many of these oxidants also damage the Hgb molecule and lead to the formation of Heinz bodies (see Hemolytic Disorders, Section C.1.a), or cause eccentrocyte and pyknocyte formation.
  B. Diagnosis of methemoglobinemia
    1. Spot test: A drop of blood is placed on white filter paper. Methemoglobinemic blood has a brown discoloration compared to blood containing oxyhemoglobin if about 10 % or more of the Hgb is metHgb.[334] Venous blood is quickly oxygenated when exposed to air, becoming redder, whereas metHgb remains brownish.
    2. CO-oximeter: This instrument uses polychromatic light to detect oxyhemoglobin, deoxyhemoglobin, carboxyhemoglobin, and metHgb by means of their different absorption spectra. The results are expressed as a percentage of total Hgb, e.g., 20 % metHgb (see Analytical Concepts, Section IV, in Chapter 10).
    3. Pulse oximeter: This device does not specifically detect metHgb, and when metHgb is present, $_spO_2$ (the percentage of Hgb saturated with O_2 based on pulse oximetry) is unreliable (see the Analytical Concepts section in Chapter 10). The $_spO_2$ is based on the different absorptivities of oxyhemoglobin and deoxyhemoglobin at red and infrared wavelengths. MetHgb absorbs similarly at both wavelengths, and with more than about

30 % metHgb, $_spO_2$ becomes fixed at about 85 %, thus overestimating the actual $_spO_2$. However, in the absence of impaired oxygenation of plasma, this $_spO_2$ would be significantly lower than the $_sO_2$ determined by blood gas analysis. This is because the $_sO_2$ is calculated directly from $_pO_2$(a) and does not reflect the actual decrease in O_2 saturation of Hgb caused by methemoglobinemia. When $_sO_2$ is substantially greater than $_spO_2$, methemoglobinemia should be considered.

4. Differentiation from sulfhemoglobin:[335] Sulfhemoglobinemia may be present with or without methemoglobinemia, though it is rarely recognized. It similarly interferes with pulse oximetry and may not be distinguishable from metHgb by some CO-oximeters. Sulfhemoglobin forms when sulfur binds irreversibly to heme in Hgb and oxidizes Fe^{2+} to Fe^{3+}, e.g., with sulfur-containing toxicants. Affected heme groups cannot carry oxygen, but unaffected groups within the Hgb tetramer release O_2 more easily. Sulfhemoglobin is not associated with decreased RBC survival; it disappears as affected RBCs slowly undergo senescent removal. Whenever there is a saturation gap between $_spO_2$ and $_sO_2$(a) that along with apparent cyanosis does not resolve with methylene blue treatment for suspected methemoglobinemia, sulfhemoglobin should be considered.

II. Cytochrome-b_5 reductase (Cb_5R) (NADH-methemoglobin reductase, EC 1.6.2.2) deficiency[336–340]
   A. Cb_5R catalyzes the primary reaction involved in the conversion of metHgb to Hgb. It uses FAD as a cofactor (Figure 3.5). A Cb_5R deficiency allows metHgb to accumulate in RBCs.
   B. Classic features, when apparent, are signs of hypoxia (exercise intolerance, lethargy) because of the poor O_2-carrying capacity of metHgb, dark to chocolate-colored blood, cyanosis, methemoglobinemia, and often Heinz bodies. There is typically no anemia, but mild anemia may be present, as might compensatory reticulocytosis or secondary appropriate erythrocytosis. Diagnosis may occur incidentally (e.g., at surgery).
   C. The disorder has been diagnosed in young and adult animals of pure (e.g., pit bull terriers, Pomeranians) or mixed breeding. Associated mutations in *CYB5R* have been described in dogs and cats.

III. Congenital methemoglobinemia in a mixed-breed dog with a promoter deletion and mutation in the gene encoding cytochrome b_5[341]
   A. This 4-yr-old castrated male dog was chronically cyanotic with methemoglobinemia, mild erythrocytosis, and laboratory evidence of functional Cb_5R.
   B. Genetic analysis revealed mutations supporting defective cytochrome b_5, the electron-transferring heme-binding protein involved in metHgb reduction by Cb_5R.

IV. Familial methemoglobinemia associated with GR deficiency in horses[330]
   A. GR catalyzes the conversion of glutathione disulfide to GSH. It uses FAD as a cofactor (Figure 3.5). A deficiency in GR can lead to a decreased [GSH] in RBCs, thus making them more susceptible to oxidants.
   B. A young mare and her dam both were exercise intolerant and had similar abnormalities. Clinical features were methemoglobinemia, mild anemia (Hct near 25 %), GR deficiency (about 30 % of activity of healthy horses), and reduced [GSH] (about 50 % of healthy values), but Cb_5R activity and NADPH dehydrogenase activity were not decreased.

V. Hereditary stomatocytosis
   A. Hereditary stomatocytoses have been recognized in Alaskan malamutes, Drentse patrijshonds, and schnauzers, and stomatocytosis has been reported in Pomeranians, beagles, and Australian cattle dogs. The specific RBC defects are not established; clinically, the dogs have different disorders, but osmotic fragility is increased in each.
   B. In the Alaskan malamutes, there is a concurrent chondrodysplasia.[342] The RBC disorder is characterized by mild anemia, mild to moderate reticulocytosis, mild to moderate stomatocytosis, and shortened RBC life span.
   C. In the Drentse patrijshonds, the concurrent findings include hypertrophic gastritis, polycystic renal disease, pelvic limb paresis, and icterus with regenerative macrocytic hypochromic anemia due to hemolysis.[130] Studies of RBC membranes and plasma of affected dogs indicate that abnormal fatty acid composition in plasma phospholipids leads to abnormal phospholipid composition in RBC membranes.[343]
   D. Miniature and standard schnauzers or schnauzer crosses with mild to marked stomatocytosis are asymptomatic and typically nonanemic with mild or no reticulocytosis, but their RBCs are macrocytic and hypochromic with increased $[Na^+]$, $[K^+]$, and $[Mg^{2+}]$, and increased membrane ion channel activity supportive of defective cation exchange.[131,132,344] Pseudohyperkalemia may be present because of in vitro K^+ leakage from RBCs, particularly with delayed sample analysis of whole blood or delayed removal of plasma or serum. The amount of stomatin (RBC membrane protein 7.2b) in RBCs of standard schnauzers with stomatocytosis was variable but similar to the amount found in dogs without stomatocytosis.[345] Mutations associated with overhydrated cation-leak stomatocytic hemolytic anemia in people were not detected in affected standard schnauzers.[344]
   E. Asymptomatic stomatocytosis similar to that seen in schnauzers has been reported in rare Pomeranians, beagles, and Australian cattle dogs.[346]

VI. Hereditary band 3 deficiency
   A. Japanese black cattle homozygous for this autosomal dominant disorder have retarded growth, metabolic acidosis, and chronic spherocytic hemolytic anemia with erythroid hyperplasia but not blood reticulocytosis.[128] These abnormalities occur because *RBC band 3* is a membrane protein that (i) serves as an ion-exchange protein for the rapid exchange of Cl^- and HCO_3^- to allow the transport of $CO_{2(g)}$ from tissues to lungs, and (ii) contributes to the RBC cytoskeleton.
   B. A nonanemic adult Jack Russell terrier had persistent reticulocytosis (about 3–10 %), intermittent mild spherocytosis, increased RBC osmotic fragility, and intermittent lethargy associated with RBC membrane deficiencies in band 3 and α- and β-adducin proteins when compared to a single healthy control dog.[347] This proteomics pattern is found in people with band 3 deficiency and hereditary spherocytosis.

VII. Hereditary elliptocytosis of dogs due to protein band 4.1 deficiency[120,348]
   A. Elliptocytosis is caused by qualitative and quantitative defects of RBC membrane protein 4.1, a protein that is needed to stabilize the cytoskeleton of the RBC membrane by providing an anchor for the spectrin-ankyrin complex. Note: A protein 4.1 variant may explain the decreased deformability and elliptical shape of camelid RBCs.[349]
   B. Classic features of the canine disorder are marked elliptocytosis and slight anemia with moderate reticulocytosis (compensated anemia). In cases reported so far, the disorder was not a major problem for the affected dogs.

VIII. Elliptocytosis in a mixed-breed dog due to mutant spectrin[121]

A. Elliptocytosis was caused by a mutation in the spectrin gene and was associated with an increased ratio of spectrin dimers to spectrin tetramers in RBC membranes.

B. Spectrin, a major protein of the RBC cytoskeleton, is composed of heterodimers of two proteins: α-spectrin and β-spectrin. These proteins self-associate to form tetramers. Molecular defects in either α-spectrin or β-spectrin can reduce this association, and thus more dimers are present in the membrane. In people, the location of the mutation in either α-spectrin or β-spectrin produces elliptocytosis or spherocytosis.[350] Elliptocytosis occurs when there is a defective horizontal interaction of membrane proteins, whereas spherocytosis occurs when the vertical interaction of membrane proteins is defective.

C. This defect was clinically silent. The elliptocytosis was subclinical, and no anemia was present. Many RBCs were involved but to a varying degree, with about 40 % type I elliptocytes (nearly circular cells), 35 % type II elliptocytes (oval cells), and 25 % type III elliptocytes (elongated cells).

IX. Spectrin deficiency in Dutch golden retrievers

A. Spectrin is a membrane protein that is a major component of RBC cytoskeletons. In people, spectrin deficiency causes hereditary spherocytosis, a disorder characterized by chronic hemolytic anemia and increased RBC osmotic fragility.

B. A partial spectrin deficiency was detected in a group of Dutch golden retrievers after they recovered from hemolytic anemias (three immune mediated and two of unknown mechanisms).[351] It was discovered because of the persistence of increased RBC osmotic fragility. The RBC [spectrin] in the affected dogs was 50–65 % of the concentrations found in golden retrievers without altered RBC osmotic fragility. After recovery from the hemolytic anemias, the dogs with partial spectrin deficiency were not anemic. Spherocytes were detected only in dogs diagnosed with IMHA. A cause-and-effect association between spectrin deficiency and hemolytic anemia was not established in these dogs. Pedigree analysis indicated an autosomal dominant inheritance.

X. Sideroblastic anemia in dogs[79]

A. A diagnosis of sideroblastic anemia was based on the concurrent presence of ringed sideroblasts and > 15 % atypical sideroblasts in marrow.

B. Of the seven dogs, six had nonregenerative anemia, one had a regenerative anemia, five had hypochromic anemias, and two had microcytic anemias. Dysplastic features in marrow reportedly included dyserythropoiesis (asynchronous nuclear maturation, binucleation, and nuclear fragmentation or lobulation), asynchronous maturation in megakaryocytes, and dysmyelopoiesis (giant neutrophils and hypersegmentation).

C. The siderocytes and sideroblasts indicated defective Fe metabolism and were associated with several disorders (acute hepatitis, pancreatitis, glomerulonephritis, and sepsis) or concurrent abnormalities (myelofibrosis). Fe stores in marrow were considered increased or within limits of health.

LABORATORY METHODS FOR ASSESSING IRON (Fe) STATUS

Evaluation of an animal's Fe status may be helpful in confirming an Fe-deficient or Fe-overload state, but serum or plasma [Fe] alone is an unreliable reflection of body Fe stores.

I. Serum or plasma [Fe]
  A. Analytical concepts
    1. Because nearly all Fe in nonhemolyzed serum or plasma is bound to transferrin, serum or plasma [Fe] measured by clinical assays typically represents the amount of Fe bound to transferrin. Fe in ferritin may contribute significantly to serum or plasma [Fe] if hyperferritinemia is marked.[352]
    2. Unit conversion: µg/dL × 0.01791 µmol/µg × 10 dL/L = µmol/L (SI unit)
    3. Stability: Data are scarce, but serum and plasma [Fe] appear to be stable for at least several hours at room temperature and for weeks if the serum or plasma is refrigerated or frozen. It is best to separate the plasma or serum within hours.
    4. Common principle of serum Fe assays: Fe^{3+} is liberated from transferrin in an acidic environment and then reduced to Fe^{2+} by ascorbic acid. Fe^{2+} reacts with a dye or other compound to form a colored complex that is detected by photometric methods. Assay modifications allow measurement of [Fe] in heparinized plasma.
  B. Hyperferremia (increased [Fe] in serum or plasma) (Table 3.16)
    1. Excess Fe intake or uptake
      a. Iatrogenic
        (1) Excess ingestion of a hematinic in calves[353] and ingestion of a digestive inoculate (Primapaste) in foals[354] have produced Fe toxicosis. In adult horses, excessive Fe content of drinking water has also caused Fe overload with increased transferrin saturation (> 80 %), hepatic fibrosis, and Fe accumulation in hepatocytes and macrophages.[355]
        (2) Fe injections may be administered inappropriately to animals suspected of being Fe deficient, especially to racehorses.[356] If Fe cannot be excreted fast enough by urine and feces, hyperferremia develops.
        (3) Transfusion of old packed RBCs causing intravascular hemolysis[357]
        (4) Many serial transfusions of RBCs causing Fe overload
      b. Ineffective erythropoiesis, as occurs with congenital dyserythropoietic disorders, PIMA, or some myelodysplastic syndromes, may contribute to hyperferremia. In these conditions, anemia leads to increased [Epo] and thus an increase in erythroid

Table 3.16. Disorders and conditions that cause hyperferremia

Fe overload due to excess intake or uptake
Iatrogenic: excess Fe injections, oral hematinics, transfusion of RBCs (old RBCs or many RBC transfusions)
Ineffective erythropoiesis: congenital dyserythropoietic disorders, PIMA, some myelodysplastic syndromes
Genetic defect in regulation of Fe absorption
Glucocorticoid administration (horses and dogs)
Release of Fe from tissues
Hepatocyte damage
Hemolysis
Hyperferritinemia

Note: Serum [Fe] can be falsely increased with hemoglobinemia as discussed in the text. Serum [Fe] in neonatal foals was high compared to adults but returned to adult values by day 3.

precursors and increased production of erythroferrone (not yet documented in domestic mammals) by the expanded population of erythroid precursors. Erythroferrone then suppresses hepcidin production, thus increasing uptake of Fe from the intestine.[358,359] Persistent ineffective erythropoiesis can cause Fe overload because of continued Fe uptake. This might also occur with the marked erythropoiesis of PK deficiency.[291]

c. Hereditary hemochromatosis was reported in cattle of the Salers breed.[360] In people, primary hemochromatosis occurs because intestinal absorption of Fe is not appropriately regulated.

2. Increased glucocorticoid hormones: Hyperferremia occurs after administration of dexamethasone in horses ([Fe] doubles within 2–3 d)[361] and in dogs,[362] but hypoferremia occurred in cattle. The reasons for changes in [Fe] are not known.
3. Release of Fe from tissues
   a. Hepatocytes typically contain Fe-rich ferritin. When hepatocyte damage occurs, the Fe that is released may cause hyperferremia.
   b. Intravascular and extravascular hemolytic states are associated with hyperferremia. Fe released from degraded Hgb binds to transferrin and is transported in plasma for reutilization or storage.
4. Hyperferritinemia (Table 3.20): Fe liberated from ferritin can cause hyperferremia when determined by some Fe assays.[352] The contribution of ferritin Fe to serum [Fe] appears to be minimal or up to about 10 % without hyperferritinemia, but possibly 20–50 % when serum [ferritin] is increased.
5. False increases:
   a. The Fe in plasma Hgb (when hemoglobinemia is present) has little influence on serum or plasma [Fe] because Fe is bound tightly to Hgb and generally not released for detection, even with acidic dye-binding methods. However, release may be greater in aged samples with denatured Hgb that binds Fe less avidly.
   b. Hgb molecules may cause spectral interference and therefore erroneous measurements (false increases in most assays).
6. Neonatal foals had serum Fe concentrations > 400 µg/dL, but the [Fe] decreased to adult concentrations (about 125–130 µg/dL) by 3 d of age.[85]
7. Other factors that likely affect [Fe] are diurnal variations, diet-dependent postprandial increases, and fasting, but little data are available for domestic mammals.

C. Hypoferremia (Table 3.17)
1. Fe deficiency
   a. Increased Fe loss caused by chronic external blood loss
      (1) Because each mL of blood contains about 1 mg of Fe, blood loss results in a loss of Fe from the body. Hypoferremia may develop when Fe stores become depleted from chronic blood loss.
      (2) Causes of blood loss that result in Fe deficiency include blood-sucking intestinal parasites, fleas and ticks, chronic gastrointestinal hemorrhage (neoplasms, ulcers), and excessive blood donation.
      (3) Acute blood loss is not expected to cause hypoferremia because Fe is mobilized from storage and carried in the plasma by transferrin.
   b. Decreased Fe intake or uptake from the intestine
      (1) Veal calves are purposely fed an Fe-deficient diet that can produce Fe deficiency.
      (2) Extensive intestinal mucosal disease that leads to impaired absorption of Fe contributes to an Fe-deficient state.

Table 3.17. Disorders and conditions that cause hypoferremia
Fe deficiency
Increased Fe loss: chronic external blood loss*
Decreased Fe intake or uptake from the intestine
Fe-deficient diet (veal calves)
Extensive intestinal mucosal disease
Iron-refractory iron-deficiency anemia (rare)
Shift of Fe from plasma*
Inflammation, acute or chronic
Renal disease
Cancer
Other or unknown mechanisms
Dexamethasone injections in cattle
Dogs with congenital portosystemic shunts
Young animals (foals, kittens, calves)*

* A relatively common disorder, condition, or mechanism

(3) Iron-refractory iron-deficiency anemia, a disorder caused by a mutation in the gene (*TMPRSS6*) encoding a downregulating enzyme (matriptase 2) of hepcidin transcription, may have been identified in a cocker spaniel.[363] In this disorder, persistently increased hepcidin impairs Fe uptake from enterocytes to plasma, leading to Fe deficiency that cannot be corrected by oral Fe administration.[364]

2. Shift of Fe from plasma
   a. In acute and chronic inflammatory diseases, serum [Fe] may be decreased because of the sequestration of Fe in macrophages. IL-6 promotes the synthesis of hepcidin, and thus the amount of membrane ferroportin available for the export of Fe from cells (including macrophages and enterocytes) is reduced. This decreases Fe transport from enterocytes to blood and limits recycling of Fe from senescent RBCs engulfed by macrophages. Hypoferremia can develop within several hours.
   b. With renal disease, inflammation-induced increases in hepcidin synthesis and decreased renal hepcidin excretion may contribute to hypoferremia.
   c. With some cancers, hypoferremia may be promoted by increased hepcidin expression by neoplastic cells or by hepatocytes secondary to inflammatory cytokines from the neoplastic cells or inflammatory cells.
   d. The altered state of Fe kinetics that causes hypoferremia in these disorders has been called a pseudo–Fe deficiency, an Fe-restricted state, or a functional Fe deficiency. In this condition, blood may be deficient in Fe, but the body is not.
3. Glucocorticoids in cattle caused hypoferremia ([Fe] dropped from ~140 µg/dL to ~50 µg/dL) within 1 d after dexamethasone (2 mg) was given intravenously.[365]
4. Portosystemic shunts in dogs
   a. Dogs with congenital portosystemic shunts may have a microcytic anemia, and some may have concurrent hypoferremia.[84,366] Serum [Fe] decreased in a group of 16 dogs with surgically induced portosystemic shunts (from a mean of 129 µg/dL to a mean of 92 µg/dL), but each serum [Fe] remained WRI.[367]

b. The pathogenesis of the hypoferremia is not established, but data support the concept of defective transport of Fe because of decreased production of transferrin by hepatocytes.[84,366]

5. Young animals
   a. Stabled Dutch warmblood foals (1–3 mo of age) that were fed freshly cut grass had lower blood [Hgb], Hct, blood [Fe], and percentage transferrin saturation than similar foals raised on pasture. The data provided evidence of Fe deficiency in the stabled foals.[368] Relative to adult MCV values, foals have a peak microcytosis at 3–5 mo of age, but their serum Fe concentrations were equal to or greater than concentrations found in healthy adult horses.[85]
   b. Of the 2- to 4-wk-old kittens in a specific-pathogen-free colony, 70 % had hypoferremia (relative to adult concentrations) that was associated with microcytosis.[87] This suggests a transient Fe-deficient state during early growth.
   c. In 18 dairy calves (< 3 d old) with apparent congenital hypoferremia, serum Fe concentrations were lower in calves with Hct values < 25 % than in those with Hct values > 25 %. Only the most severely anemic calf (Hct = 9 %) had clinical signs of anemia. The cause of the hypoferremia and Fe-deficient state was not determined.[92]
6. Disorders that cause a loss (e.g., proteinuria) or decreased production of plasma proteins, including (apo)transferrin, might lower plasma [Fe], possibly enough to cause hypoferremia.

II. TIBC and UIBC
   A. Analytical concepts
      1. Terms and units
         a. *TIBC*, a measure of plasma capacity to carry Fe, is the maximum concentration of Fe that can be bound by plasma or serum proteins. Because most plasma Fe is in transferrin (apotransferrin complexed with Fe), serum [Fe] depends on and correlates with serum [transferrin]. A transferrin molecule can contain two Fe^{3+} ions, but Fe^{3+} occupies only about a third of all plasma transferrin Fe-binding sites in health.
         b. *UIBC*, a measure of the total unused (open) Fe-binding sites on transferrin, is the additional [Fe] that could be protein bound in the sample, a reflection of [transferrin].
         c. Unit conversion: μg/dL × 0.01791 μmol/μg × 10 dL/L = μmol/L (SI unit)
      2. Sample: Serum may be preferred, but plasma may be acceptable. Serum [TIBC] is stable for a few days if the sample is refrigerated or kept at room temperature, and it is stable for months if frozen.
      3. Principles of serum or plasma TIBC and UIBC assays
         a. Direct immunoassays for transferrin are marketed for some domestic mammals but validation studies were not found.
         b. Common method
            (1) UIBC is determined by adding a known amount of excess Fe to alkalinized serum or plasma to saturate all Fe-binding sites on transferrin. The unbound Fe is then measured by absorption after it complexes with a chromogen. The difference between the added amount of Fe and the measured endpoint unbound [Fe] represents the UIBC. A lower endpoint [Fe] reflects more open Fe-binding sites on transferrin, so a greater UIBC.
            (2) TIBC is calculated: TIBC = UIBC + [Fe]. Because the serum or plasma [Fe] almost entirely reflects transferrin-bound Fe, it is largely a measure of [transferrin].

Table 3.18. Disorders and conditions that cause increased TIBC
Increased apotransferrin production
Fe deficiency (see the text for species variations)
Other or unknown mechanisms
Several conditions reported in dogs (see the text)
Young animals (foals)

Note: TIBC is lower in greyhounds than in nongreyhound dogs.

c. Alternative method: Excess Fe is added to saturate transferrin and the excess unbound Fe is adsorbed away. Fe is dissociated from transferrin by acid, reduced, and complexed to a chromogen so that the amount of Fe that was bound to transferrin can be measured. This is the TIBC, which can be used to calculate the UIBC: UIBC = TIBC – [Fe].

d. In some clinical assays, plasma Hgb (hemoglobinemia) may cause spectral interference in assays that measure [Fe] (see Laboratory Methods for Assessing Iron Status, Section I.B.6) and thus could result in erroneous TIBC and UIBC results.

B. Increased serum TIBC (Table 3.18)

1. Fe deficiency: Fe-deficient dogs typically do not have TIBC greater than upper reference limits, and decreased concentrations have been reported,[84,362] but TIBC increased as nutritional Fe deficiency developed in dogs,[369] and increases have been documented with Fe deficiency in calves, foals, and people.
2. Fe overload: Few TIBC data have been reported for domestic mammals with Fe overload, but increases have been reported in cattle and dogs.[360,370] Measurement of TIBC concurrently with [Fe] allows calculation of percent transferrin saturation, which is increased in Fe overload.
3. In one study, TIBC was increased in some dogs with IMHA, hepatic disease, histiocytic sarcoma, and lymphoma.[371] Many of these dogs were hyperferritinemic, but specific mechanisms of increased TIBC were not explored.
4. Young foals, especially near 1 mo old, have much greater TIBC (> 600 µg/dL) than neonates or adult horses. Colostrum is transferrin rich, but colostral intake does not explain the entire increase in TIBC during a foal's first month of life.
5. Greyhounds have lower TIBC than nongreyhounds.[372]

C. Decreased serum TIBC (Table 3.19)

1. Decreased transferrin production
   a. Inflammation: Apotransferrin is a negative acute-phase protein (i.e., its production is decreased by actions of inflammatory mediators such as IL-1), so inflammation may lead to hypotransferrinemia.

Table 3.19. Disorders and conditions that cause decreased TIBC
Decreased apotransferrin production
Inflammation*
Hepatic insufficiency, including portosystemic shunts
Malnutrition (possibly)
Increased transferrin loss (protein-losing states)

* A relatively common disorder, condition, or mechanism

b. Hepatic insufficiency, including portosystemic shunts: Because transferrin is a β globulin produced by hepatocytes, hepatic disease or shunting that causes hypoproteinemia may cause hypotransferrinemia. Hypotransferrinemia may play a role in the microcytosis that develops in some animals with hepatic failure or portosystemic shunts.[84]
c. Malnutrition may contribute to decreased transferrin production.
d. Congenital atransferrinemia and congenital hypotransferrinemia have not been reported in domestic mammals but are rare causes of microcytic hypochromic anemia, hypoferremia, hemosiderosis, and hyperferritinemia in people.

2. Increased transferrin loss: Severe glomerular lesions causing protein-losing nephropathy may cause hypotransferrinemia. The relative molecular mass of transferrin is only slightly greater than albumin's. Other protein-losing states (e.g., severe protein-losing enteropathy and hemorrhage) could also decrease serum TIBC because of transferrin loss.

III. Percent transferrin saturation (% saturation)
A. Analytical concepts
1. Percent transferrin saturation is a calculated estimate of the percentage of Fe-binding sites on apotransferrin molecules that are occupied by Fe.
2. Percent transferrin saturation = serum or plasma [Fe] × 100 ÷ TIBC
B. Changes in % transferrin saturation depend on changes in [Fe] and TIBC.
1. Greater % transferrin saturation occurs when serum [Fe] is increased, TIBC is decreased, or both.
2. Lower % transferrin saturation occurs when serum [Fe] is decreased, TIBC is increased, or both.

IV. Stainable Fe in macrophages of marrow, spleen, or liver
A. Hemosiderin is present in macrophages. It decreases with Fe deficiency and increases with increased total body Fe or Fe sequestration.
B. In formalin-fixed tissue or unstained air-dried cytologic preparations, hemosiderin is a yellow-brown granular or globular material in macrophages.
C. When attempting to quantify the amount of Fe in storage, an Fe-selective stain (e.g., Prussian blue) enables a more definitive assessment than do routine stains. However, the process is subjective and requires knowledge of how much Fe is expected in tissues of healthy animals.
D. The marrow of healthy cats does not contain stainable Fe, so feline marrow cannot be used to assess Fe stores. Fe stores are also undetectable or minimal in healthy young, growing animals of any species.

V. Serum ferritin concentrations
A. Analytical concepts
1. Unit conversion: ng/mL × 1000 mL/L × 1 μg/1000 ng = μg/L (SI unit)
2. Sample: Serum or plasma can be used. Ferritin concentrations appear to be stable for at least 7 d at 2–8 °C and for many months at –20 °C.
3. Principles of serum ferritin assays: Species-specific immunoassays are used to measure ferritin in dogs, cats, and horses.[85,373]
B. Hyperferritinemia (Table 3.20)
1. Increased apoferritin production

Table 3.20. Disorders and conditions that cause hyperferritinemia
Increased apoferritin production
Increased total body Fe
Iatrogenic: excess injected Fe, oral Fe, or blood transfusions
Ineffective erythropoiesis: congenital dyserythropoietic disorders, PIMA, some myelodysplastic syndromes
Genetic defect in regulation of Fe absorption
Hemolytic disease, likely (increased Fe availability)*
Inflammation*
Neoplasms:* histiocytic sarcoma
Hemophagocytic syndrome (potentially)
Shift of ferritin from tissue to plasma
Hepatic disease*
Exercise in horses

* A relatively common disorder, condition, or mechanism

a. Increased total body Fe: Increased total body Fe increases intracellular Fe which stimulates increased production of apoferritin. This may occur from (i) excess Fe administration orally, parenterally, or by transfusions containing RBCs, (ii) ineffective erythropoiesis associated with PIMA, congenital dyserythropoietic disorders, or some myelodysplastic syndromes (likely related to increased erythroferrone and hence decreased hepcidin production),[359] or (iii) genetic defects in the regulation of Fe absorption.[360]
   (1) If neither inflammation nor hepatic disease is present, serum or plasma ferritin concentrations in people are correlated with Fe storage.[374] If Fe toxicity is present, the typical ferritin assay measures tissue ferritin released from damaged cells, and thus the amount of Fe storage is overestimated.
   (2) In a group of 95 dogs, there was a weak but statistically significant correlation (r = 0.37) between serum [ferritin] and nonheme-Fe content of liver and spleen, but none of the assessed dogs had increased Fe stores.[375] A similar correlation (r = 0.365) was present in 106 random-source cats.[373]

b. Hemolytic disease (likely): Intracellular Fe and therefore apoferritin production might increase with increased Fe availability due to Fe recycling in hemolytic disorders. Hyperferritinemia tends to be greater in dogs with IMHA than with other inflammatory disorders or neoplasms.[371] With hemolysis form PK deficiency, increased Fe uptake might contribute to Fe availability because of chronically increased erythropoiesis and erythroferrone production.

c. Inflammation: Because apoferritin is a positive acute-phase protein, cytokine (e.g., IL-1) stimulation of hepatocytes may cause hyperferritinemia. In people, the Fe saturation of ferritin decreases with inflammation.[376] Inflammation may contribute to hyperferritinemia in some patients with hemolytic anemias, e.g., IMHA.

d. Neoplasms: Hyperferritinemia has been considered a marker for several human neoplasms and may be caused by inflammation, accelerated RBC turnover (hemolysis), or increased production by neoplastic cells. Hyperferritinemia has been associated with histiocytic sarcomas in dogs.[371,377]

e. As reported in people, hyperferremia may occur in some reactive hemophagocytic syndromes.
f. Horses with hyperinsulinemia may develop hyperferritinemia through dysregulation of Fe homeostasis, perhaps by impaired hepcidin function (not deficiency).[378] Fe overload occurs with dysmetabolic hyperferritinemia in a subset of human patients with type II diabetes mellitus.

2. Shift of ferritin from tissue to plasma
   a. Hepatic disease such as hepatitis and necrosis: Tissue ferritin is released from damaged hepatocytes and is measured by ferritin assays that do not selectively measure glycosylated (or plasma) ferritin.[374] Most clinical assays probably measure glycosylated and nonglycosylated ferritin.
   b. Plasma ferritin concentrations in horses increased with exercise. The exact mechanism for the increased [ferritin] is not established, but ferritin appears to be released from tissues other than liver.[379] Samples for the assessment of Fe stores should be drawn at least 2 d after any strenuous exercise.

C. Hypoferritinemia
1. Decreased Fe storage
   a. Serum or plasma ferritin concentrations decrease as Fe stores decrease.
   b. In people, ferritin concentrations decrease early in Fe deficiency and before decreased blood [Hgb], microcytosis, or decreased serum [Fe] is detected. Once Fe stores are depleted, plasma [ferritin] remains low.
2. Because of opposing processes, an Fe-deficient animal with inflammatory disease may not be hypoferritinemic.

VI. Decreased reticulocyte Hgb content (CHr or RET-He) and volume (MCVr)

A. RET-He and CHr both assess the Hgb content of reticulocytes, but by different methods; the correlation between canine RET-He and CHr was only fair ($r = 0.74$) in one study.[47] Because RET-He measurement is affected by RBC volume irrespective of RBC Hgb content, it may be falsely increased by macrocytosis and falsely decreased by microcytosis.

B. Decreased CHr, RET-He, and MCVr can occur with Fe deficiency or insufficient Fe availability, both of which impair Hgb synthesis and erythropoiesis.[47,192] These decreases reflect the state of erythropoiesis within just the last 3–4 d. They may occur with early or mild disease (e.g., overused blood donors)[380] prior to or without changes in MCV, MCH, MCHC, and CHCM, values that mostly reflect the more numerous circulating cells produced earlier. Later correction of CHr, RET-He, and MCVr supports a return to appropriate erythropoiesis.

C. Insufficient Fe availability (Fe restriction) occurs with Fe sequestration or because Fe cannot be transferred to erythroid precursors fast enough to meet the needs of increased erythropoiesis.
1. Increased inflammatory cytokines associated with inflammation or neoplasms may cause hepcidin-mediated Fe sequestration and decreased CHr, RET-He, and MCVr,[153] changes that may precede anemia.
2. Some dogs with IMHA have decreased CHr that reflects inadequate Fe availability; this may relate to increased need because of erythroid hyperplasia or to impaired mobilization because of inflammation.[381]
3. Portosystemic shunts (and likely hepatic failure) may cause decreased MCVr, CHr, and RET-He, potentially by impaired production of transport proteins.[84]

Table 3.21. Comparative Fe profile results

	Serum [Fe]	Serum TIBC	Stainable Fe in marrow[a]	Serum [ferritin]
Fe deficiency	↓	WRI–↑	↓	↓
Inflammation	↓	WRI–↓	WRI–↑	WRI–↑
Overload of Fe due to excess intake (diet or iatrogenic)	↑	?, may ↑	↑	↑
Increased glucocorticoids (except cattle)	↑	?	?	?
Pathologic hemolysis (in vivo)	WRI–↑[b]	WRI–↑[b]	WRI–↑	↑
Young animals (vs mature)	↓ (foals and kittens)	↑ (foals)	↓	↓
Hepatic insufficiency	?	WRI–↓	?	↓–↑[c]

[a] Marrow samples of healthy cats do not have stainable Fe.
[b] Plasma Hgb may cause erroneous values (see the text).
[c] [Ferritin] could be decreased because of decreased production or it could be increased because of hepatocyte damage.

4. Therapy with recombinant Epo may require Fe administration to provide enough available Fe to maximize erythropoiesis; decreased CHr, RET-He, and MCVr would signal insufficient Fe availability with Epo therapy.

D. MCVr and consequently CHr and RET-He may be decreased with breed-related hereditary microcytosis; smaller cells contain less Hgb, even with appropriate Hgb synthesis.[84]

VII. Comparative Fe profile results

A. Assessment of the Fe status of an animal is enhanced if the laboratory assays are grouped as a profile.

B. As shown in Table 3.21, the two major causes of hypoferremia – Fe deficiency and inflammation – can be differentiated by the assessment of Fe stores (either by stainable marrow or serum ferritin concentrations). However, other conditions or disorders that can alter serum [Fe], TIBC, and Fe stores need to be considered.

BLOOD TYPING AND CROSSMATCHING

I. Blood typing

A. Blood groups (see Physiologic Processes, VII. Blood groups or types) are important for transfusion medicine and NI.

1. If transfused RBCs are recognized as having foreign surface antigens, these are the two major consequences:
   a. If there are circulating antibodies in the recipient, those antibodies can attach to the transfused RBCs and mediate hemolysis.
   b. If there is not a circulating antibody in the recipient, the recipient's immune system may be triggered to produce one.

2. Anti-RBC alloantibodies or isoantibodies may be naturally occurring (i.e., present without previous exposure), or acquired if they develop after exposure to RBC antigens from another member of the species. *Xenoantibodies* are antibodies in one species that target RBC epitopes in a different species; naturally occurring anti-RBC xenoantibodies are common.
3. In neonates with NI, hemolysis is mediated by colostral alloantibodies or isoantibodies that are absorbed and then bind to paternally derived epitopes on a neonate's RBCs.

B. Blood typing involves various methods to detect the RBC blood-group factors on the surface of the RBCs. Most methods involve antibodies that bind to the factors. Monoclonal antibodies are available for identifying some blood-group antigens, but most typing reagents have been polyclonal serum antibodies produced by alloimmunization and subsequent removal of unwanted RBC-reactive antibodies by adsorbing them to RBCs of other blood types. Blood typing is dependent on availability of typing reagents, some of which are unavailable or limited to a few specialized laboratories. However, in-clinic blood typing kits have been developed for some blood types, and these kits continue to evolve.

C. The various blood typing methods have different strengths (advantages) and weaknesses (disadvantages). They do not yield results with 100 % agreement, and some results are not clear. If unclear results cannot be assessed by a more confirmatory method in a timely manner, compatibility decisions should include consideration of the potential effects of using incorrectly typed blood products.
1. RBCs from donors with false-negative blood-group factor results could be immunogenic for recipients that are negative for the antigen. To avoid immune-mediated transfusion reactions, donors with equivocal results are best considered positive (not used for negative recipients). High diagnostic sensitivity (few false-negative results) is important for donor testing.
2. Recipients with false-positive blood-group factor results might be administered RBCs that are immunogenic. To avoid this, recipients with equivocal results are best considered negative and given only negative RBCs. High diagnostic specificity (few false-positive results) is important for recipient testing.

D. Agglutination methods: Many blood typing methods rely on antibody- or lectin-induced RBC agglutination to signal the presence of a particular blood-group factor. RBC autoagglutination prevents blood typing by these methods unless washed RBCs no longer autoagglutinate.
1. Tube method: Washed RBCs are incubated with typing reagent in test tubes before assessing for reagent-mediated agglutination indicative of positivity for the factor in question. An antiglobulin reagent may be required for some antibodies. Autoagglutination is assessed in control tubes. Although long the standard for blood typing, tube typing is relatively labor intensive and not standardized with respect to typing reagents, dilutions, washing, incubation temperatures and times (may vary with antibody), and even what constitutes a positive reaction (1+ or 2+ agglutination).
2. Card (or slide) methods: Blood-typing cards have been developed commercially for canine and feline blood. A drop of whole blood in buffer is added to a white card impregnated with an antibody or other agglutinin, e.g., *Triticum vulgaris* lectin (wheat germ agglutinin) for feline factor B. If there is a positive reaction, the RBCs agglutinate, but agglutination can be equivocal. If autoagglutination of RBCs is present in the card's control region, agglutination in the test well cannot be interpreted, but washing of the RBCs may allow testing if washing eliminates agglutination.

a. For dogs, a blood-typing card is used to determine whether a dog is DEA 1 positive or negative. The agglutinin is a monoclonal antibody to DEA 1 only. A diluent is added to reconstitute the antibody. If agglutination occurs in the positive control and patient regions, the dog is positive for DEA 1. Reassessing a negative result by addition of more diluent might overcome a prozone reaction occurring when the ratio of antibody to antigen does not favor agglutination. A similar card test based on polyclonal antibodies has been developed for Dal typing; results may be unreliable if the Hct is < 20 %, but sample Hct can be adjusted by centrifugation. A murine monoclonal antibody to Dal has been developed and is likely to be used for card typing.
b. For cats, a blood-typing card is used to determine whether a cat is type A, B, or AB. Type A antigen is detected with an agglutinating antibody; type B antigen has been detected with a monoclonal antibody or a lectin. Some reactions have been weak or inapparent, either because of the method or because of low expression of the A antigen secondary to genetic variants in *CMAH*, the gene responsible for its formation.[32] Confirmation of type AB results by another method has been recommended. Confirmation of type B results can be done be assessing for naturally occurring anti-A alloantibodies by crossmatching (so-called backtyping).

3. Cartridge method: Canine DEA 1 and feline factors A and B have been assessed by a single-use cartridge and analyzer system that eliminates user evaluation for agglutination. Whole blood is added to a diluent tube based on the sample Hct, and the mixture is drawn into the cartridge where it moves along by capillary action. The fluid reconstitutes monoclonal antibodies or a lectin within the cartridge, and agglutination is detected by increased light transmission through the clear spaces that form in the moving fluid as RBCs agglutinate. An algorithm assesses the pattern of light transmission to yield a positive, negative, or inconclusive result.
   a. For DEA 1 testing, the sample is split into two channels. In one, RBCs mix with a monoclonal antibody to DEA 1, while the other is used to assess for autoagglutination.
   b. For feline A and B testing, the blood is split into two channels and assessed for agglutination by using monoclonal antibodies or a lectin.
   c. Samples with high or low Hct may need Hct adjustment prior to testing (kit directions should be followed). Hemolysis can interfere by altering light transmission.
4. Gel column method: Small gel columns built into plastic cards contain a reaction chamber over either a neutral gel or gel containing blood typing antibodies, lectins, or an antiglobulin reagent. A dilute suspension of RBCs in assay buffer is dispensed into the reaction chamber, with or without serum or antibodies, depending on the test. After incubation, if needed, the card undergoes low-force centrifugation in a special centrifuge to force single RBCs through the column to form a pellet at the bottom. RBCs agglutinated by anti-factor antibodies are retained above or within the gel, thus signifying positivity for a blood group antigen. Results are not interpretable if RBCs are retained in the control gel column without the addition of antibodies; this can happen with autoagglutination or with a suspension of RBCs that is too concentrated. The gels are stable for days and can be photographed for a permanent record. They have been used for blood typing dogs and cats.[382,383]

E. Immunochromatographic methods: Individual RBCs from a diluted sample of whole blood or packed RBCs migrate up an absorbent membrane strip. RBCs accumulate in a red band if they express the antigen to which a line of antibodies or lectins incorporated onto the

membrane bind. To interpret the results, a positive control band must form further up the strip where a reagent that binds to any RBC is incorporated. Results can be captured by imaging. For AB cats, red bands would form at the levels of anti-A, anti-B, and control reagents. Weak bands have been reported with low-Hct samples, but RBCs can be concentrated for such samples. Results are not expected to be affected by autoagglutination if enough RBCs are individualized and therefore able to migrate and form visible bands.

F. Flow cytometry: Flow cytometric assays have been developed with polyclonal or monoclonal antibodies to detect specific blood group factors such as DEA 1 and feline A and B. IgG-coated RBCs may be used as a positive control.[384]

G. Genetic testing: This can be used to assess AB status of cats and may be useful for breeding decisions, avoidance of NI, and troubleshooting equivocal or contradictory immunohematologic typing results.[385] However, it does not replace immunohematologic typing and crossmatching.

II. Crossmatching

A. Blood typing cannot be done for unknown antigens or for all known antigens to ensure blood type compatibility, so crossmatching is done to assess for RBC incompatibilities without identifying them specifically. Crossmatching is typically used to help determine whether an animal's blood can be safely and effectively transfused into another animal. The main clinical concern is that an incompatible major crossmatch could induce an acute hemolytic transfusion reaction whereby recipient alloantibodies mediate the acute destruction of transfused RBCs. Crossmatching is similar to blood typing in that the laboratory assays involve antibody reactions to RBC antigens. However, they are different in that the antibody is present in a recipient's plasma (for a major crossmatch) or in the donor's plasma (for a minor crossmatch).

B. Crossmatching may be done as a precaution prior to transfusion in any species. It has been advocated for all cats and horses because of the frequency of clinically relevant naturally occurring antibodies, particularly if they received prior transfusions, and it is recommended for dogs that have been transfused at least 4 d prior (or at all), have an unknown transfusion history, or had a recent transfusion reaction.[386,387] Unlike the situation for people and horses, pregnancy does not appear to sensitize dogs to RBC antigens.[218]

C. The basic aspects of the assays are as follows:[388]

1. Blood is drawn from a patient that needs a blood transfusion (the recipient) and from a donor. Plasma or serum is separated from the RBCs, and the RBCs are washed in saline several times to remove loosely bound proteins. If EDTA-plasma is used, chelation of divalent cations by EDTA inhibits complement and therefore assessment of complement-mediated hemolysis. Results may differ in fresh and stored equine samples,[387] but potential donor canine RBCs can be stored for about a month.
2. Major crossmatch
   a. The procedure involves mixing and incubating a dilute suspension of potential donor's RBCs with the recipient's serum or plasma. Antibody binding is detected by agglutination, hemolysis, or a DAT (antiglobulin-enhanced crossmatching). Agreement among methods has varied from very good to poor depending on the methods and study.
      (1) Tube (or microtiter plate) agglutination method: Although considered the gold standard method, tube typing is labor intensive, not standardized (e.g., for dilutions, incubation times and temperatures, macroscopic versus microscopic assessment), and can have equivocal endpoints. Autoagglutination interferes.

(2) Gel agglutination method:[389] Similar to gel blood typing, centrifugation of gel columns overlain by a mixture of donor RBCs and recipient serum or plasma in buffer can be used to detect agglutinates retained by the gel. Retained RBCs indicate an incompatibility if autoagglutination or marked rouleaux are not present. Alternatively, with an antiglobulin-enhanced crossmatch, washed RBCs from an incubated mixture of RBCs and serum or plasma can be mixed with an antiglobulin reagent in the gel column's incubation chamber prior to centrifugation. This can enhance detection of incompatibilities.
A complement source can be added to assess for hemolysis, but the sensitivity to detect hemolysis may be lower than with other methods.[390] Negative control samples can consist of RBCs with autologous plasma or serum; positive control samples of IgG-coated RBCs can be created by incubating typing serum with type-positive cells. Modifications of a canine method may be needed to overcome problems caused by the greater rouleau formation of equine samples.

(3) Immunochromatographic method:[389] Donor RBCs incubated with recipient plasma or serum are washed, pelleted, and resuspended in migration buffer prior to placing the end of the immunochromatographic strip into the suspension so that RBCs migrate up the strip. A red control band formed by binding of any RBCs to a reagent indicates adequate migration of RBCs, and a red band at the level of an antiglobulin reagent indicates the presence of IgG, IgM, and/or C3. Donor RBCs incubated with donor plasma or serum and then the antiglobulin reagent must yield no antiglobulin band for a test band to be interpretable.

b. A compatible major crossmatch indicates that antibodies against the donor's RBCs were not detected, thus making an immune-mediated hemolytic transfusion reaction less likely. However, some clinically significant antibodies are not detected by agglutination and require complement reactions or a DAT for detection.

c. An incompatible major crossmatch indicates that antibodies against the donor's RBCs were detected, thus indicating that a transfusion of RBCs may induce an immune-mediated hemolytic transfusion reaction. However, incompatible crossmatches may occur when antibodies are not clinically significant.

3. Minor crossmatch (not done as frequently as the major)

a. The procedure involves mixing and incubating a dilute suspension of recipient's RBCs with potential donors' sera or plasma. Tube, gel, and immunochromatographic methods have been used.

b. An incompatible minor crossmatch is usually not a clinical concern because it is not expected to cause a major immunologic transfusion reaction.

III. Species variations

A. Naturally occurring antibodies against DEA 1 or Dal have not been reported, and no naturally occurring antibodies have been shown to cause clinically apparent acute hemolytic transfusion reactions in dogs. Therefore, rejection of the first transfusion of blood of unknown type is not expected in dogs. However, clinically apparent acute hemolytic transfusion reactions may occur against DEA 1, DEA 4, Dal, or unknown blood types in previously transfused dogs that developed antibodies to these antigens. Also, some naturally occurring antibodies (e.g., to DEA 7, DEA 3) or post-transfusion antibodies might cause decreased RBC survival without a clinically apparent acute hemolytic reaction.

B. Cats have naturally occurring antibodies against the A or B antigen they are lacking, i.e., type A cats have anti-B antibodies, type B cats have strong anti-A antibodies, and type AB cats do not have either antibody. In addition, Mik-negative or FEA 1–negative cats may have naturally occurring alloantibodies to Mik or FEA 1. NonAB typing is not widely available, so use of AB- and crossmatch-compatible blood products are generally recommended, even in transfusion-naïve cats.

C. Blood-type systems in horses are more complex, and crossmatching procedures are not as simple as those for dogs and cats. Some of the antibodies are agglutinins, but others are poor agglutinins and thus require complement as a reagent to detect antibody binding via hemolysis.[391]
   1. About 10 % of horses have naturally occurring alloantibodies to a RBC antigen they lack; their transfusion with crossmatch-incompatible RBCs based on agglutination may result in shortened RBC survival.[34]
      a. Horses lacking RBC antigen A may have anti-Aa or anti-Ac antibodies.
      b. Horses lacking RBC antigen Ca typically have a low titer of anti-Ca antibodies.
      c. Antibodies to RBC antigens D, K, P, and U are rare to infrequent.
   2. Horses may be sensitized to RBC antigens via blood transfusions or during pregnancy or parturition. The acquired antibodies that cause transfusion reactions or NI are primarily against A, Q, and C. The antibodies may cause an incompatible reaction in the major crossmatch; blood from a pregnant mare and stud can be crossmatched prior to parturition to assess for risk of NI.

D. Blood-type systems in cattle are extremely complex, and thus it is essentially impossible to give a compatible transfusion.

E. Information focused on crossmatching of alpaca or llama blood was not found.

METHODS TO DETECT RBC SURFACE ANTIBODY OR COMPLEMENT

I. Purpose: To detect ESAIg (mostly IgG or IgM) or C3 on a patient's RBCs, usually to help diagnose IMHA, particularly in patients with nonagglutinating, nonspherocytic, regenerative anemias without a clear cause; the test may be unnecessary when there are clinical and laboratory findings that strongly support primary or secondary IMHA, although it might be useful for confirmation or follow-up assessment.

II. Tube or microtiter plate DAT (Coombs test)[392,393]
   A. Analytical principles: Analysis generally involves the following steps, but there are many method variations related to wash times, wash buffers, antiglobulin reagents, antiglobulin concentrations, incubation temperatures, endpoint evaluation (macroscopic only or also microscopic), and use of positive control samples.
      1. Sample: EDTA-anticoagulated blood is preferred. Canine samples have given reproducible positive or negative results when stored for 7 d at 4 °C.[59] If RBC agglutination appears to be present in the blood tube or with a saline dispersion test, it may disappear with washing, thus allowing testing.
      2. Estimated 2–5 % RBC suspensions are prepared from anticoagulated patient blood (EDTA or ACD anticoagulation) and washed 3–4 times with 0.9 % saline or phosphate-buffered saline to remove unbound proteins.
      3. In tubes or, more efficiently, in microtiter plate wells, RBCs are incubated with serial dilutions of antiglobulin reagent (species-specific anti-IgG, anti-IgM, and/or anti-C3).

Reagents that contain two or more types of antiglobulin are *polyvalent*, whereas reagents specific for just one globulin are *monovalent*. Antiglobulins bind to IgG, IgM, or C3 on the washed RBCs. Addition of saline rather than antiglobulin reagent serves as a negative control. With autoagglutination of the negative control sample, test results are invalid. Autoagglutination is evidence of a pathologic or nonpathologic agglutinin from an in vivo or in vitro source.

4. Samples are incubated at 4 °C, room temperature, and/or 37 °C prior to centrifugation and assessment for agglutination. Agglutination may be assessed as soon as 5 min and/or after 30–60 min of incubation. A source of complement proteins may be added to assess for hemolysis, which is needed for equine testing because reagent antibodies may not induce RBC agglutination even when bound to their target antigens on RBCs. Positive control samples can consist of RBCs coated with immunoglobulin by incubation with an appropriate blood typing reagent.
5. Positive tube reactions are recognized by the presence of RBC agglutinates as RBC pellets are resuspended; the degree of agglutination is graded. Positive plate reactions are recognized by formation of a diffuse matte in the well bottom, whereas negative wells contain condensed buttons that stream when the plate is tilted. The temperatures and reagent titers at which agglutination occurred are reported for each antiglobulin reagent used, polyvalent and monovalent. Samples may be positive with one or more reagents at one or more temperatures.
    a. Agglutination indicates that a patient's RBCs are coated with at least hundreds of immunoglobulins or C3 molecules and that the binding of the antiglobulin immunoglobulin was sufficient to cause agglutination (Figure 3.24).

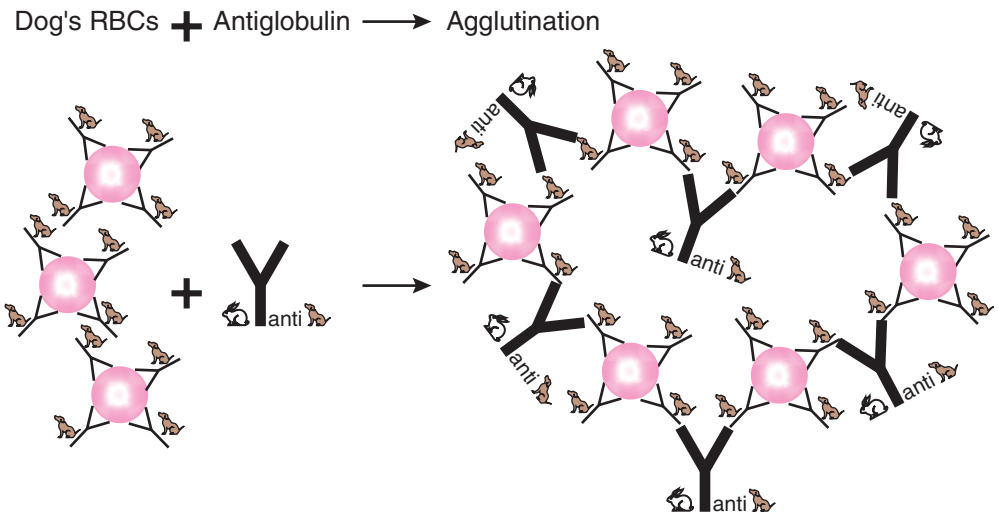


Figure 3.24. Agglutination reaction of a positive canine direct antiglobulin (Coombs) test. Washed canine RBCs are incubated with species-reactive antibodies to IgG, IgM, and/or complement C3, shown here as rabbit anti-(dog IgG). The dog's washed RBCs are coated with dog IgG, so the antiserum causes agglutination of the dog's RBCs when the concentration of rabbit anti-(dog immunoglobulin) is appropriate and allows lattice formation. Agglutination is a positive result. Coombs tests may be designed to detect each immunoglobulin and complement separately (monovalent test) or together (polyvalent test).

(1) For agglutination to occur, there must be the appropriate ratio of antigen and antibodies to form an agglutinating lattice; with too many antiglobulin immunoglobulins, each antibody tends to bind to a separate antigen rather linking RBCs together by binding to target antigens on each of them. This can cause a *prozone reaction*, a false-negative result (no agglutination) at lower reagent dilutions (reagent antibody excess).

(2) Serial dilutions of antiglobulin reagent are used in tube and plate methods to vary the antigen to antibody ratio and reduce false-negative results caused by antibody excess. With a prozone pattern, the DAT would be negative with a low dilution but positive at greater dilutions, e.g., negative at a 1:2 dilution of the antiglobulin reagent, weakly positive at a 1:4 dilution, and strongly positive at a 1:8 dilution.

b. Hemolysis suggests that immune complexes had formed between antiglobulin antibodies and their targets, thus leading to complement activation and RBC lysis.

B. Interpretation

1. Results must be interpreted in light of other clinical and laboratory findings. Samples from some healthy or sick animals without clinically significant hemolysis yield positive results, and negative results occur in some patients that appear to have IMHA.
2. A positive DAT in an anemic patient supports an immune-mediated hemolytic component to the anemia but does not confirm it or establish a diagnosis.
   a. The significance of positivity at various temperatures is not clear. Some dogs diagnosed with primary IMHA have had DAT results for IgG that were negative at 37 °C and positive at 4 °C,[394] but positivity only at 4 °C or room temperature may not reflect findings in vivo at body temperature.
   b. After initiation of immunosuppressive glucocorticoid therapy, positive results support IMHA but negative results do not exclude it.[59]
   c. A positive post-transfusion result might relate to the transfusion rather than underlying disease, although many post-transfusion results are negative in dogs with nonimmune anemias.[59]
   d. Positive reactions (matte formation) reported in negative-control wells for feline samples tested at 4 °C may relate to a high prevalence of cold agglutinins or a species difference in RBC dispersal with the plate method.[395,396] IMHA was found to be unlikely in cats with samples that do not autoagglutinate at 4 °C and are DAT-negative at 37 °C, but positivity at 37 °C was not specific for IMHA and occurred, though rarely, in nonanemic cats.[396]
3. Negative DAT results indicate that nonimmune causes of anemia should be considered. However, negative results may occur in samples from patients with immune-mediated hemolysis when (i) the number of IgG, IgM, and/or C3 proteins is too low to detect, (ii) hemolysis is mediated by an unassessed antibody (e.g., IgA), or (iii) any of several analytical errors occurs. IMHA-inducing antibodies are not expected to be removed from RBCs by washing, but the potential for removal of low-avidity antibodies or adherent nonRBC antigen with bound antibody has not been completely excluded.

III. Flow cytometric direct antiglobulin test (DAT)

A. Analytical principles

1. EDTA blood is the preferred sample. There is evidence that blood may be stored at 4 °C for up to 7 d before testing.[203]
2. After RBCs are washed with saline or phosphate-buffered saline, they are incubated with fluorochrome-labeled antibodies against IgG, IgM, IgA, or C3. If the patient's

RBCs are coated with antibodies or C3, the fluorochrome-labeled antibodies bind to them to make those RBCs fluoresce.

3. After washing, flow cytometric analysis identifies those RBCs that are fluorescent and those that are not. A positive result has been identified by an increased percentage of fluorescent cells[397,398] or by an increased mean or median fluorescence intensity of the RBC population.[14] The method of determining positivity can greatly impact the results, especially for mildly positive samples.
4. RBCs coated with antibody by incubation with appropriate typing sera may be used as positive control samples. Patient samples incubated without labeled antibodies and patient samples incubated with nonsense antibodies can be used as negative-control samples.

B. Disorders with increased ESAIg or C3
1. Finding RBCs coated with increased ESAIg or C3 supports a conclusion of immune-mediated hemolysis but does not establish a diagnosis. Any of the immune-mediated hemolytic disorders can be considered, including idiopathic IMHA, drug-induced IMHA, vaccine-induced IMHA, NI, incompatible blood transfusion, and IMHA induced by bacterial and viral infections. False positive results might occur with in vitro (but not in vivo) binding of target globulins (e.g., cold-reactive IgM) or various analytical errors.
2. Negative results might occur if hemolysis is mediated by an unassessed antibody type (e.g., IgA) or there are analytical errors, including those related to criteria for positivity. The potential for washing to remove low-avidity antibodies (not expected) or adherent nonRBC antigen with bound antibody has not been completely excluded.

IV. Other DAT methods used in veterinary medicine have included immunochromatographic strip, capillary tube, and gel microcolumn methods, the latter two being based on agglutination.[59] The immunochromatographic strip functions similarly to strips used for blood typing, but rather than being based on agglutination, an antiglobulin reagent in the strip captures RBCs bearing the target globulins.

V. In contrast to direct tests for ESAIg, indirect antiglobulin tests assess patient serum or plasma for anti-RBC antibodies by incubation with test RBCs. Indirect tests are most often used for detection of alloantibodies. If available, they have much lower diagnostic sensitivity than DATs for IMHA, likely because of low plasma antibody titers (antibodies rapidly bind to RBCs).[399] False negative results are possible if patient antibodies target molecules adherent to RBCs or other epitopes that are not universally present on RBCs and therefore not on test RBCs. A positive indirect antiglobulin test with a negative DAT may occur because of alloantibodies generated by prior transfusion or, in some species, by naturally occurring alloantibodies.

OSMOTIC FRAGILITY TESTING

I. RBC *osmotic fragility* is the susceptibility of RBCs to lysis in hypoosmotic solutions. In such fluids, RBCs are relatively hyperosmotic, which leads to H_2O entry, swelling, and lysis. Increased osmotic fragility is associated with decreased RBC survival and therefore certain hemolytic anemias. RBCs that are less susceptible to osmotic lysis have increased *osmotic resistance*. Osmotic fragility is distinct from *alkaline fragility*, the susceptibility to lysis at alkaline pH.

II. Indications: Osmotic fragility testing is used in clinical and investigational settings to provide evidence for or against a hemolytic component to anemias of unclear pathogenesis.[311,400] However, increased osmotic fragility is nonspecific and occurs with several hemolytic conditions; it has also been reported in patients without other clear clinical evidence of hemolysis.

III. Analytical methods
- A. Sample
  1. Whole blood should be collected into heparin or EDTA tubes, depending on a laboratory's protocol. Sample osmolality and results may vary with the anticoagulant and ratio of anticoagulant to sample.
  2. Fragility generally increases with sample age, but changes appear to be mild within the first 2 d of storage at 4 °C.[401,402] Mild decreases in fragility have been reported, perhaps because the most fragile subpopulation lysed in storage before testing.[76] Concurrent testing of a similarly handled sample from a healthy animal can be done to help assess for potential sample changes during shipment or storage.
- B. Effects of incubation pH, time, and temperature have been described.[403] With the classical osmotic fragility test, standard volumes of whole blood (15–30 μL) are added to standard volumes (1–5 mL) of phosphate-buffered saline (typically pH 7.4) in a series of 13–19 tubes containing from 0.9 % or 0.85 % NaCl down to 0 % NaCl. Some investigators have normalized Hct to a standard value. After incubation at a standardized temperature and for a standardized time, commonly at room temperature for 30 or 45 min (but some protocols use 37 °C for 24 h), supernatant is harvested by centrifugation, and the optical densities are determined spectrophotometrically at 540 nm to detect Hgb. The percentage of maximal hemolysis in each tube is calculated by comparing absorption of each supernatant to the absorbance in the 0 % NaCl (H_2O) supernatant (all RBC lysed). A fragility curve (fragiligram) may be generated by graphing % hemolysis against saline concentration, usually from high to low (left to right). This allows determination of concentrations causing various degrees of hemolysis from initiation to completion, e.g., 5 %, 10 %, 50 %, or 90 % hemolysis, and evaluation of the shape of the curve and its shift to the left or right. A derivative curve can also be generated by graphing the incremental changes in hemolysis at each [NaCl]; this can facilitate recognition of RBC populations with different fragilities.
- C. A test variation is to assess hemolysis at just two saline concentrations (e.g., 0.9 % and 0.55 %) with comparison to full lysis in H_2O. With this quicker test, a positive result for increased fragility is a pink or red color of the supernatant at the lower [NaCl] but not at the higher concentration;[400] sample hemolysis prior to testing impairs interpretation. Flow cytometric methods have also been developed.

IV. Major interpretive considerations
- A. Expected findings in health: When graphed with the highest saline concentration to the left, results yield a sigmoid curve with a steep central slope through the *median corpuscular fragility (MCF)* or *median osmotic fragility (MOF)* – the [NaCl] aligning with 50 % hemolysis (sometimes denoted NaCl50) – and with the lower and upper tails plateauing to 0 % and 100 % hemolysis at the high and low NaCl concentrations, respectively. A reverse sigmoid curve is generated when NaCl concentrations run from low to high (left to right) on the x axis.
- B. Osmotic fragility varies with species, generally increasing with decreasing MCV in mammals with discoid RBCs,[404] and being lowest in camelids,[405] whose thin elliptocytes can become spheres in hypotonic fluid.[406]

C. Anemia alone has not been demonstrated to affect fragility results; no association with Hct was seen over the broad range (18–19 percentage points) of Hct in healthy dogs and cats.[76] Potential effects of anemia are limited by comparing degrees of hemolysis to the total possible hemolysis for the same sample.
D. Increased osmotic fragility of the majority of the RBCs is apparent when the sigmoid curve shifts toward the higher [NaCl] (to the left with typical graphing but to the right with a reverse sigmoid curve) and the MCF is increased, indicating that milder degrees of hypoosmolality induced lysis. Loss or diminution of the lower plateau (more sloped) without an overall left shift of the curve reflects a subpopulation of RBCs with increased fragility.
E. Decreased osmotic fragility (increased osmotic resistance) is reflected by a shift of the curve toward lower NaCl concentrations (a lower MCF), and a subpopulation of osmotically resistant RBCs may make the upper tail of the curve less flattened.

V. Disorders associated with increased osmotic fragility (Note: If many of a patient's fragile RBCs have been lysed in vivo during an acute hemolytic event, sample RBCs might not reflect the fragility that was present.)
A. IMHA:[400] Increased osmotic fragility is commonly present in IMHA and has been attributed largely to spherocytosis; the decreased surface area to volume ratio of spherocytes compared to discocytes is expected to limit the degree to which RBCs can take up H_2O before lysing. Concurrent oxidative damage may play a role.
B. Idiopathic hemolytic anemia with splenomegaly (osmotic fragility syndrome) in cats: A constellation of findings first described in Abyssinian and Somali cats includes moderate to marked osmotic fragility.[310] Similar findings have since been described in other cat breeds and in domestic shorthair cats.[311] The cause or causes of anemia in these cats is unknown.
C. Hereditary stomatocytosis: Alaskan malamutes, Drentse patrijshonds, and schnauzers; stomatocytoses has also been reported in Pomeranians, a beagle, and an Australian cattle dog
D. Hereditary membrane protein deficiencies
1. Band 3 deficiency in Japanese black cattle[128] and a Jack Russell terrier[347]
2. Spectrin deficiency in Dutch golden retrievers
3. Hereditary nonspherocytic hemolytic anemia in beagles; a membrane defect was suspected[308,309]
E. Dyslipidemias:[96,400,402] Changes in blood lipoproteins can alter RBC membrane lipid content and yield poikilocytes and/or increased osmotic fragility. Increased osmotic fragility has been reported in dogs with diabetes mellitus, in dogs receiving glucocorticoids, and, experimentally, with diet-induced hypercholesterolemia. The degree to which various dyslipidemias promote in vivo hemolysis is unknown, but lipemia-induced hemolysis is commonly seen in vitro.
F. Oxidative RBC injury: Induction of a Heinz body hemolytic anemia by administration of an oxidant was associated with increased osmotic fragility,[407] but no association was detected between osmotic fragility and the number of Heinz bodies in healthy cats with up to 56 % of RBCs containing Heinz bodies.[76] Eccentrocytes and pyknocytes are expected to have increased osmotic fragility because of decreased surface area to volume ratios.
G. Infectious hemolytic anemias: Increased osmotic fragility was reported in dogs with *Babesia canis* infections,[408] and in an anemic cat with a hemotropic mycoplasma infection.[76]
H. Envenomation: Osmotic fragility may be increased after certain envenomations.
I. Increased RBC osmotic fragility has been reported in mongrel dogs with high-K and high-glutathione RBCs[409]and in a shar pei with high-K RBCs (glutathione was not assessed).[410]

J. Other: Increased RBC osmotic fragility has been documented in individual animals with a variety of other inflammatory and neoplastic disorders, but associations between the disorders and the fragility are not clear. Although osmotic fragility may be mildly increased in some individuals with RBC enzymopathies including PK deficiency, it is often WRI.

VI. Disorders associated with decreased osmotic fragility: Although testing is generally done for evidence of increased osmotic fragility in patients with potential hemolytic disorders, increased osmotic resistance also occurs. It may be expected when RBCs have increased surface area to volume ratios as occurs with reticulocytosis, codocytes in several disorders (e.g., Fe deficiency, portosystemic shunts), or with persistent hyponatremia. Osmotic resistance has been noted with cholestasis and cholestatic dyslipidemias[402,411] and leptospirosis (potentially related to hepatic or renal disease).[412]

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 4

PLATELETS

Michael A. Scott and Steven L. Stockham

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Table 4.1. Abbreviations and symbols in this chapter

[x]	x concentration (x = analyte)
ATP	Adenosine triphosphate
CBC	Complete blood count
CD	Cluster of differentiation
CTAD	Citrate, theophylline, adenosine, and dipyridamole
DIC	Disseminated intravascular coagulation
EDTA	Ethylenediaminetetraacetic acid
Fc	Fragment (crystallizable)
Hct	Hematocrit
Ig	Immunoglobulin (A, E, G, or M)
IL-6	Interleukin 6
IMHA	Immune-mediated hemolytic anemia
IMT	Immune-mediated thrombocytopenia
IPF; IPF%	Immature platelet fraction; immature platelet fraction percentage
MPC	Mean platelet component
MPM	Mean platelet mass
MPS	Mononuclear phagocyte system
MPV	Mean platelet volume
PCDW	Platelet component distribution width
PDW	Platelet distribution width
PSAIg	Platelet surface–associated immunoglobulin
QBC	Quantitative buffy coat
RBC	Red blood cell, erythrocyte
RI	Reference interval
RNA	Ribonucleic acid
RPlt; RPlt%	Reticulated platelet; reticulated platelet percentage
SI	Système international d'unités (International System of Units)
SLE	Systemic lupus erythematosus
Tpo	Thrombopoietin
WRI	Within reference interval

PHYSIOLOGIC PROCESSES

I. Mammalian platelets are small cytoplasmic fragments of megakaryocytes (*mega* = "large," *karyon* = "nucleus," and *cyte* = "cell") that are derived from pluripotent hematopoietic stem cells (Figure 2.1). They are also called *thrombocytes*, the term used for the analogous nucleated cells in birds, reptiles, and amphibians. Mammalian platelets share most of the following components, although with species variations:
   A. A phospholipid membrane containing glycoproteins important in cell-cell interactions and phospholipids important for coagulation
   B. An open canalicular system of membrane invaginations that serves as a conduit for substances moving between platelets and plasma, and that contributes to a marked

increase in external platelet surface area after activation; it is not developed in horses, cattle, or camels, whose granules must fuse with the cell membrane for secretion[1]

C. A cytoskeleton, associated with a peripheral microtubular ring and abundant actin and myosin, which maintains platelets in a discoid shape and allows for shape change and pseudopod formation with activation

D. A dense tubular system of endoplasmic reticulum that stores Ca^{2+} for platelet activation and that is important in thromboxane synthesis

E. α-Granules that store numerous proteins involved in hemostasis and vessel repair and whose contents are secreted with appropriate stimuli

F. Dense granules containing Ca^{2+}, Mg^{2+}, adenosine diphosphate, ATP, and serotonin, all of which are secreted with appropriate stimuli

G. Glycogen stores and mitochondria for energy

II. Mammalian platelet production: The process of generating circulating platelets from hematopoietic stem cells requires megakaryopoiesis and thrombopoiesis. Adequate megakaryopoiesis is reflected by adequate numbers of megakaryocytes in the bone marrow but does not guarantee adequate thrombopoiesis. (Thrombocyte production in birds, reptiles, and amphibians does not involve megakaryocytes.)

A. *Megakaryopoiesis*, the proliferation and maturation of megakaryocytes, occurs in hematopoietic tissue, mostly bone marrow but commonly spleen as well. Resident myeloid progenitor cells respond to cytokines – primarily Tpo – by undergoing proliferation, differentiation, and maturation.[2,3] Megakaryocytes increase their ploidy to as much as 128n (128× the haploid number of chromosomes) and expand their cytoplasmic volume through repeated *endomitosis*, which is mitosis without cell division or nuclear segregation via anaphase. Limited baseline megakaryocyte production occurs in the absence of Tpo, yielding platelet concentrations 10–15 % of normal.[4]

B. *Thrombopoiesis*, the formation of platelets from megakaryocytes and their delivery to the circulation, appears to be independent of Tpo, but Tpo is the main regulator of platelet production through its effects on megakaryopoiesis.[2–4] A single megakaryocyte produces thousands of platelets.

1. In the bone marrow, megakaryocytes begin development in the osteoblastic (endosteal) niche and then migrate to the vascular niche (perisinusoidal) to complete their maturation. A variety of matrix molecules and cytokines produced by local osteoblasts, endothelial cells, fibroblasts, adipocytes, macrophages, megakaryocytes, and other cells create each microenvironment.
2. CXCL12 (stromal cell-derived factor 1) and fibroblast growth factor 4 are two chemokines produced by marrow cells that bind to receptors on cells of the megakaryocyte lineage and help direct them to the vascular niche where they interact with endothelial cells.
3. Once megakaryocytes have matured, they extend pseudopods, also called proplatelets, through or between endothelial cells in response to high vascular concentrations of sphingosine-1-phosphate. The pseudopods elongate and partition into strings of interconnected cytoplasmic beads that are released as platelets in the marrow sinusoids or in circulation after proplatelets are released from their parent megakaryocyte. Some whole megakaryocytes enter sinusoidal blood and become trapped in the lung, in the first small vessels they encounter (Figure 4.1).
4. The lungs appear to play an important role in thrombopoiesis. Some data suggest that over 50 % of platelets are produced from megakaryocytes and proplatelets

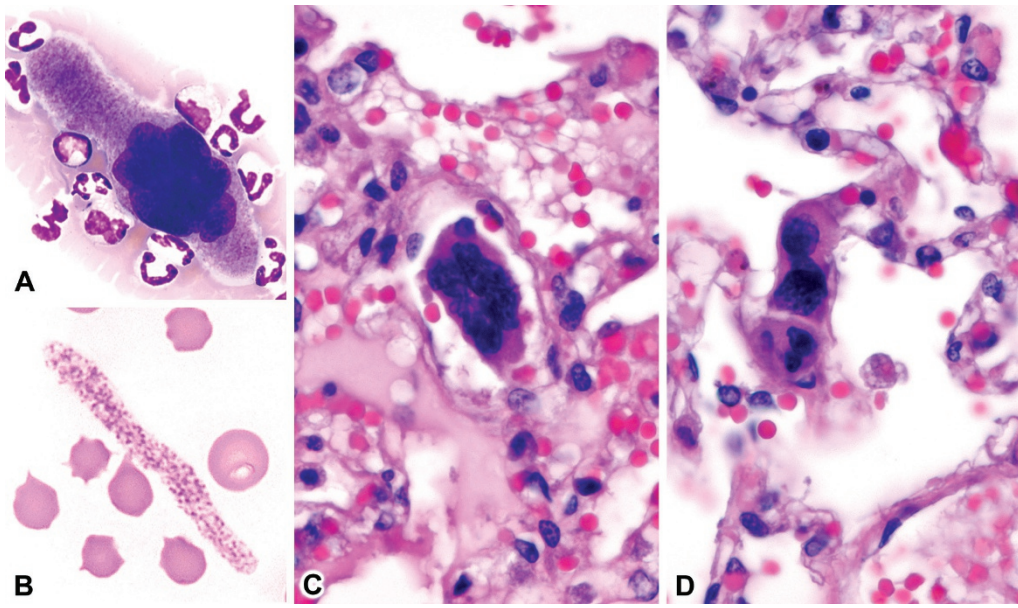


Figure 4.1. A. Megakaryocyte in the feathered edge of a blood film, dog. B. Proplatelet in bovine blood. C and D. Megakaryocytes (center of each frame) released from bone marrow produce platelets when trapped in pulmonary vasculature, fixed cat lung, hematoxylin and eosin stain.

trapped within the lungs.[5,6] Turbulent blood flow and specific interactions between vessel walls and megakaryocytes or their proplatelets promote platelet formation.[5] Lungs contain intravascular and extravascular megakaryocyte populations.[7] The origin of the extravascular megakaryocytes is unknown (bone marrow or local stem cells), and this population may function more as immunoregulatory cells than as platelet factories.

5. To a minor degree in health, and to a larger degree in pathologic states with increased thrombopoietic stimulation, megakaryocytes may release platelets by rapid cytoplasmic fragmentation without proplatelet formation.[2]

C. Tpo is produced primarily by hepatocytes, but other cells, such as osteoblasts, might produce Tpo in certain states.[8] Tpo mediates its effects through the Tpo receptor (CD110) that is often referred to as c-mpl because it is the proto-oncogene related to the murine myeloproliferative leukemia virus oncogene; the receptor was discovered before Tpo was identified as its ligand. The receptor is expressed on cells of the megakaryocyte lineage, including platelets, and on hematopoietic stem cells.

1. Although hepatocyte Tpo production was long thought to be constitutive, with plasma [Tpo] regulated mostly by total platelet/megakaryocyte mass, there is also evidence that Tpo synthesis in health is regulated by hepatic removal of senescent platelets via interactions with endocytic asialoglycoprotein receptors called Ashwell-Morell receptors and with macrophage galactose lectin on Kupffer cells.[9,10] As platelets age, deglycosylation (desialylation, removal of sialic acid) of platelet surface proteins (e.g., glycoprotein 1b) allows them to be recognized by these receptors on hepatocytes or Kupffer cells, and desialylated platelets have been shown to

accumulate within Kupffer cells.[10] It is not clear if platelet apoptosis contributes to deglycosylation, but it may. Platelet clearance through this pathway causes receptor-driven increases in hepatocyte Tpo synthesis and release, thus promoting megakaryopoiesis.

2. The overall mass of platelets plus megakaryocytes may contribute to regulation of plasma [Tpo] via receptor-mediated uptake of Tpo, there being an inverse relationship between [platelet] and blood and bone marrow [Tpo].
   a. With thrombocytosis, more Tpo binds to receptors and is removed from circulation, so there is less stimulation of megakaryocytes or stem cells.
   b. With thrombocytopenia, more Tpo remains unbound and is available to stimulate megakaryocytes. However, when megakaryocyte hyperplasia accompanies thrombocytopenia, more Tpo becomes bound by megakaryocytes and thus the plasma [Tpo] is lower than expected based solely on the [platelet].
3. Tpo promotes megakaryocytic hyperplasia (megakaryopoiesis), which leads to subsequent thrombopoiesis in bone marrow, spleen, and lungs. Hepatic Tpo is critical for hematopoietic stem cell expansion, survival, or both.[8]

D. Young platelets: At least some newly formed platelets contain increased amounts of RNA just as young RBCs (reticulocytes) do; these are analogously called *reticulated platelets*. Reticulated platelets have been shown to be less than 24 h old in dogs,[11] and they have been assessed in blood samples to gauge thrombopoiesis in dogs and horses.[1,2,12,13] Reticulated platelets are not necessarily large, but platelets formed in states of stimulated thrombopoiesis tend to be large ("stress platelets").[14]

III. Platelet kinetics

A. The blood [platelet] is generally established by the relative rates of platelet production, consumption, and destruction, and by the shifting of platelets to and from the circulation (Figure 4.2).

B. Platelet production is affected mostly by the degree of cytokine stimulation and the number of responsive cells. In rodents and people, the total megakaryocyte maturation time ranges from about 2 d to 10 d.[15]

C. Platelet life spans vary among species and are about 5–10 d in health.[16–18]

D. Platelet consumption is ongoing because of the continuous repair of minor vascular defects. This consumption is not the major determinant of platelet life span, but mean platelet life span is decreased in any markedly thrombocytopenic state, even when thrombocytopenia is caused by decreased production. This is because of a fixed platelet requirement for maintenance of vascular integrity; when marked thrombocytopenia is present, a substantially greater percentage of the circulating platelets is consumed during routine maintenance.[19]

E. In health, platelet life span appears to be determined largely by hepatic clearance of senescent platelets with desialylated surface proteins and/or apoptotic changes that may include desialylated surface proteins and phosphatidylserine expression.[20,21] Platelet apoptosis (and hence life span) depends on the balance between proapoptotic (Bax) and antiapoptotic (Bcl-xL) molecules of the intrinsic (mitochondrial) apoptotic pathway. Pharmacologic inhibitors of Bcl-xL cause platelet apoptosis and thrombocytopenia mediated primarily by the liver. However, in previous studies, splenectomized dogs had almost 50 % longer platelet life spans (8 d) than healthy nonsplenectomized dogs (about 5–6 d), supporting a role for the spleen in determining platelet life span.[16]

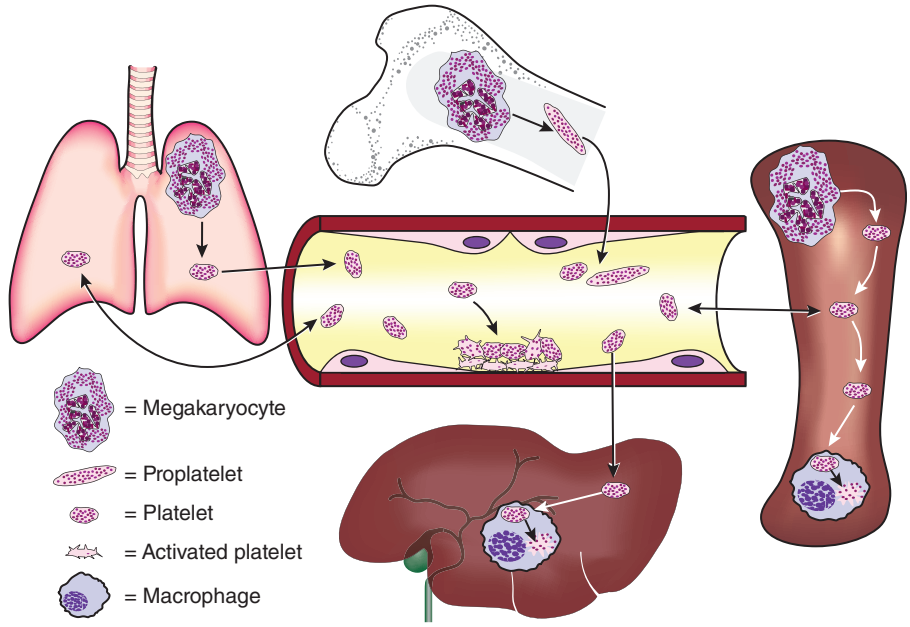


Figure 4.2. Platelet kinetics. Blood platelet concentrations are established mostly by the relative rates of platelet production, consumption, and destruction, and less so by the shifting of platelets to and from the circulation. In pathologic states, combinations of these factors often contribute to abnormal platelet concentrations.

- Production: Most megakaryopoiesis occur in the bone marrow (some in the spleen), and new platelets enter systemic circulation via marrow (or splenic) sinusoids, mostly via megakaryocyte pseudopods (proplatelets). Some megakaryocytes and proplatelets enter blood and become lodged in the lungs after release from the bone marrow. These megakaryocytes release platelets into the blood. Evidence is growing that suggests the lungs are an important source of platelets. Hematopoietic foci may also arise in the liver and contribute to the circulating platelet mass.
- Destruction: Senescent platelets become desialylated and are cleared in the liver via endocytic asialoglycoprotein (Ashwell-Morell) receptors on hepatocytes or hepatic macrophages. In disease, macrophages, primarily in the spleen and liver, also engulf platelets that carry surface-associated IgG or complement, and cytotoxic T lymphocytes may lyse them directly (not shown). Phagocytosis likely clears apoptotic or damaged platelets as well.
- Consumption: Platelets are removed from circulation during normal maintenance of vascular integrity or during accelerated consumptive states (e.g., thrombotic disease and vasculitis).
- Redistribution: Splenic platelet sequestration (reversible) reduces the circulating platelet mass, and splenic contraction increases it. Pulmonary sequestration of platelets has been associated with severe hypothermia and endotoxemia.
- Massive transfusion with blood products or other fluids may cause a dilutional decrease in platelet concentration (not shown).

F. Human and rabbit spleens have been shown to harbor about 33 % of blood platelets at any given moment.[15,22,23] Epinephrine and splenic contraction mobilize these platelets to circulation, while splenic engorgement traps more.[15,23] Splenic pooling and mobilization of platelets are expected in other species too.[23] However, 5 min after phenylephrine administration to induce splenic contraction in horses, there were significant increases in Hct (rising from a mean of 38 % to 50 %) without increases in [platelet].[24]

IV. Platelet functions (see Chapter 5 for details regarding the roles of platelets in hemostasis)
- A. The major function of platelets is to help repair vascular damage and prevent hemorrhage by participating in the formation of hemostatic plugs containing aggregated platelets. Platelets are consumed in this ongoing process.
- B. Energy for platelet functions is derived from ATP generated by aerobic glycolysis and oxidative phosphorylation. Glucose enters platelets by facilitated diffusion (GLUT transporters).
- C. Nonhemostatic platelet functions: Platelets play important roles in inflammation, immunity, wound healing, and cancer, some of which exacerbate disease. They have effects on other cells through direct contact, formation of microparticles, or release of numerous stored or newly synthesized bioactive molecules including proinflammatory mediators, cytokines, mitogens, and growth factors. They interact with WBCs and endothelium, promoting WBC transendothelial migration and WBC recruitment, neutrophil extracellular trap (NET) formation, and activation of dendritic cells and T lymphocytes. They are antigen-presenting cells. They contribute to death of organisms through release of microbiocidal substances, phagocytosis, and bacterial clearance from the circulation. Binding of bacterial lipopolysaccharide to platelet toll-like receptors can trigger release of substances that recruit inflammatory cells. Platelets also promote angiogenesis, both in wound healing and in neoplasms, and they facilitate cancer growth and metastasis.

ANALYTICAL PRINCIPLES AND METHODS

I. Thrombogram components: The quantitative and qualitative data of a hemogram or CBC that are related to platelets (thrombocytes); see Appendix A for major concepts pertaining to the CBC and blood film evaluation of platelets.
- A. Blood film evaluation
  1. Microscopic evaluation of platelets on a stained blood film is an important part of the thrombogram, especially in bleeding patients or when thrombocytopenia or extreme thrombocytosis is present.
  2. It serves three major purposes:
     - a. It enables detection of platelet clumps, which may falsely decrease [platelet] and falsely increase the WBC concentration. Some analyzers detect and report the presence of some platelet clumps, but others do not.
     - b. It enables estimation of [platelet] for corroboration of automated values or when automated measures are unavailable or unreliable.
     - c. It enables evaluation of platelets for abnormal features.
- B. Platelet concentration
  1. The *platelet concentration* is the number of platelets per unit volume of blood (in clinical jargon, commonly referred to as *platelet count*, but a platelet count is used to calculate a platelet concentration).
  2. Unit conversion: $(\# \times 10^3/\mu L) \times 10^6\ \mu L/L = \# \times 10^9/L$ (SI unit)
- C. MPV
  1. *MPV* is the mean apparent volume of all the particles in a blood sample that are counted as individual platelets, whether the particles are platelets or not and whether they are individual or in a small cluster.

2. Some analyzers additionally provide an indication of the proportion of detected large platelets, e.g., % large platelets (ADVIA) or platelet large cell ratio (P-LCR, Sysmex), using cutoffs of 12 fL or 20 fL, respectively.
3. MPV unit: fL (μm^3)

D. PDW
1. *PDW* is an assessment of platelet anisocytosis (variation in volume) calculated from the distribution of individual platelet volumes.
2. Units: % (ADVIA) or fL (Sysmex)

E. Pct (thrombocrit): platelet plus *-crit* (from *krinein*, meaning "to separate")
1. *Pct*, like Hct, is the percentage of blood volume filled by platelets (typically < 0.5 % in health). Although often referred to as the platelet mass, it is not a measure of mass or reported in units of mass; as a concentration and not a mass, it can vary with decreases or increases in plasma volume unrelated to the total number or mass of platelets in the blood.
2. Units: % or decimal form (L/L or unitless)

F. MPC and PCDW
1. *MPC* is a calculated concentration generated by ADVIA analyzers based on measured platelet refractive index (5°–15° light scatter of individual platelets) and the refractive indices of H_2O, proteins, carbohydrates, and lipids. It reflects platelet density. With activation and secretion of granule contents, MPC decreases along with platelet density, refractive index, and high-angle light scatter.
2. *PCDW* (or mean platelet component concentration distribution width) is the variation of platelet refractive index based on the standard deviation of the refractive index histogram. Increases can reflect less buffer-induced platelet sphering and thus greater scatter variation related to varied platelet orientation in the laser beam.
3. Units for each: g/dL or g/L (SI unit)

G. Analyzers may flag when platelet clumps are suspected, but significant platelet clumping may be present without samples being flagged, and these flags can be triggered when platelet clumps are not detected on smears, e.g., with lipemia.[25,26] Smear evaluation is recommended when assessing any automated platelet data.

II. Quantitative methods

A. Sample
1. EDTA anticoagulation of venous blood is routine for CBCs (see Appendix A).
   a. Citrated venous blood (1 part citrate to 9 parts blood) can be used (e.g., when EDTA-induced platelet clumping is suspected),[27] but [platelet] and certain platelet indices may be affected (see specific tests).[26] Citrate dilution requires that the measured [platelet] be corrected (corrected [platelet] = measured [platelet] × 1.1).
   b. Heparinized samples are not recommended because platelet clumping frequently occurs with heparin anticoagulation.
2. The ideal sample lacks platelet clumps, but platelet clumping is common. *Platelet clumping* is a generic term that encompasses passive agglutination and reversible or irreversible active aggregation. In veterinary medicine, most platelet clumping is thought to reflect aggregation secondary to activation. The care required to minimize platelet activation during phlebotomy and sample handling is perhaps not fully appreciated until one has substantial experience with various platelet function tests.

3. Platelets can be easily activated during blood collection, and activation causes clumping and other changes that affect platelet parameters (e.g., MPV, MPC). Platelets can clump because they are hyperactive, because of slow or poor venipuncture technique, or because of delayed or inadequate mixing of blood and anticoagulant. Clean venipuncture of a large vein (e.g., jugular) with collection directly into a vacuum tube is most likely to yield samples without significant platelet clumping. Drawing into a syringe with transfer to a tube increases the occurrence of platelet activation and clumping. Platelets of cats and cattle are very prone to clumping with routine collection methods. Just because a value is WRI when platelet clumps are present does not mean that it is accurate, as the RI is wide, and the animal may actually have a thrombocytosis. The greater the number of large platelet clumps at the feathered edge of a blood smear, the more likely the analyzer [platelet] is falsely decreased to a clinically significant degree.[25] However, there are no universal guidelines for determining this, partly because the degree of platelet clumping in a single blood smear is not completely reflective of the degree of clumping in the rest of the sample.[28]
4. The platelet inhibitor prostaglandin E1 (PgE1) or the more stable inhibitor iloprost, a synthetic analogue of prostacyclin (PgI2), can be used in very small liquid volumes to inhibit platelet activation and therefore the formation of platelet clumps. Reports suggest better inhibition of platelet clumping when platelets contact the inhibitors quickly (e.g., by having inhibitors in the EDTA tube when blood enters).[25,29] Use of these inhibitors is not practical in most settings.
5. Blood collection tubes containing citrate, theophylline, adenosine, and dipyridamole (CTAD tubes) provide platelet inhibitory factors that help reduce in vitro platelet activation.[30,31] These may be particularly useful in cats. Even the addition of EDTA-anticoagulated blood to CTAD-containing tubes promptly after collection reduced platelet clumping and its effects on [platelet] in cats.[32]
6. Addition of amikacin to EDTA tubes prior to blood collection reduced platelet clumping in feline blood samples.[33] When added post-collection to EDTA-anticoagulated blood samples containing platelet clumps, clumping decreased and platelet concentrations increased by a mean of 134 % for feline samples and 32 % for canine samples; the only other detected alteration was a mild increase in RBC mean cell volume.[34] How closely the platelet concentrations of amikacin-treated samples aligned with actual platelet concentrations was not assessed. The mechanism of amikacin's effect is unknown.

B. Platelet concentration
1. Citrate anticoagulation has caused false decreases in [platelet] attributable to platelet activation.
   a. Historically, Becton Dickinson stopped producing a partial-draw citrate tube (less vacuum and more head space to allow smaller samples in standard-sized tubes) because these tubes were associated with platelet activation during tube filling, and this could in turn cause falsely decreased platelet concentrations and altered partial thromboplastin coagulation times in samples from heparinized patients.
   b. In one study of dogs with neoplasms for which samples were collected by syringe and transferred to citrate tubes (rather than being collected directly into vacuum tubes), [platelet] was consistently low and appeared to be related to platelet activation and clumping.[26] This may have occurred during blood collection and transfer, as citrate is generally an excellent anticoagulant for use in platelet function studies.

2. For all platelet quantitation methods, platelet clumping must be absent to be confident that a [platelet] is accurate. This is best determined by evaluation of a blood smear made from a sample at the time of platelet concentration analysis. If platelet clumps are present, measured values from most analyzers can reasonably be considered minimum values because only the individualized platelets are counted and clumps do not appear to break apart enough to significantly increase [platelet] within an analyzer.[28] Although mild clumping likely has little clinically significant effect on [platelet], there is no universal definition of what constitutes mild clumping. If accurate values are required (e.g., to confirm an apparent decrease), a new sample should be collected in such a way as to minimize platelet activation and therefore clumping (see Coagulation, Section II.C, in Chapter 5).
3. There are varied statements regarding the stability of platelet concentrations.[25,35,36] Mean concentrations of groups of tested samples appear reasonably stable for 8 h at room temperature and for 24 h at 4 °C. However, concentrations may increase or decrease considerably in individual samples, so prompt testing is recommended. With room temperature storage, there may be mild to moderate decreases in canine [platelet] at 24 h and even more so at 48 h.[37–39]
4. Blood smear [platelet] estimate
    a. Approximate platelet concentrations can be calculated from estimates of the average number of platelets per 1000× field in at least 5 (preferably 10) appropriate fields (see Appendix A) by using an appropriate conversion factor: 1 platelet/1000× field = 15,000–20,000 platelets/μL.[40] For example, if the mean number per 1000× field was 4, then the estimated [platelet] would range from about 4 × 15,000 platelets/μL = 60,000 platelets/μL to about 4 × 20,000 platelets/μL = 80,000 platelets/μL. Moderate to marked thrombocytopenia and moderate to marked thrombocythemia can be recognized in this way (Figure 4.3).
        (1) The area represented by a 1000× microscopic field varies with the optics of microscopes. The diameter of the field of view varies directly with the field number of the oculars. Because of this, the appropriate conversion factor varies. The 1:20,000 conversion is more appropriate for regular oculars, and the 1:15,000 conversion is more appropriate for wide-field (22 mm) oculars. Either

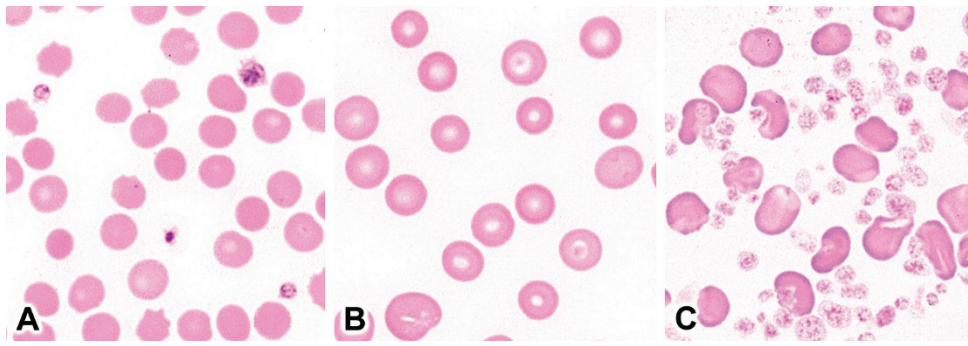


Figure 4.3. Canine blood film platelet densities representing health (A), thrombocytopenia (B), and marked thrombocytosis due to essential thrombocythemia (C, from ASVCP slide contributed by Dr. C.P. Mandell et al., 1987).

provides a useful estimate. In one of the authors' (MAS) labs, 15 clinical pathologists, residents, or laboratory technologists used wide-field microscopes to estimate platelet concentrations for eight blood films with a range of platelet concentrations from < 20,000/μL to > 1,000,000/μL; the resultant empirical conversion factor was about 14,000/μL for this group.

(2) The estimated [platelet] should be compared to species-specific RIs for interpretation. To exclude thrombocytopenia, dogs (most breeds), cats, and cattle should generally have at least 8–10 platelets per 1000× field (22 mm ocular), and most horses (and greyhounds) should have at least 5 platelets per 1000× field. Breed-specific reference guidelines are needed for greyhounds (the lower reference limit for greyhounds is lower than for most dogs and similar to that of most horses) and they may be needed for other breeds, e.g., Icelandic horses.[41]

(3) If evaluated areas are too thick, the [platelet] will be overestimated. This occurs, for example, with smears from anemic animals for which fields deeper into the body of the smear might be evaluated. In those regions, the RBC density may look appropriate because of the anemia, but the smear thickness is greater, so more platelets would have been deposited per field.

b. Another method to estimate [platelet] is to determine the ratio of platelets to either RBCs or WBCs in several fields, and then calculate the estimated [platelet] from either the RBC or WBC concentration (e.g., for 5 platelets per WBC and a [WBC] of 20,000/μL, the estimated [platelet] = 5 × 20,000/μL = 100,000/μL). These methods require quantitative RBC or WBC concentrations.

c. When estimating [platelet] from blood smears, platelet clumping should be considered. If present, the [platelet] estimation or measured [platelet] may be inaccurate, mildly so if clumping is mild and markedly so if large clumps and/or microclots are present.

5. Impedance methods (see Chapter 2 and Figure 2.4 for basic principles and analyzers)
   a. Typically, [platelet] is measured in the same path as RBC parameters and is the concentration of particles that are within a defined particle volume interval (e.g., 2–30 fL) based on the increase in electrical impedance as they pass through a narrow aperture.
   b. For most samples, a properly adjusted and calibrated impedance cell counter provides reliable platelet concentrations for most dogs, horses, and cattle, but not cats.
   c. Impedance may not differentiate small RBCs from platelets (e.g., in iron deficiency), or large platelets from RBCs (e.g., in many cats or in dogs with macrothrombocytopenia). Cats have a greater variation in platelet volume and more large platelets than other species, so impedance methods are problematic for accurately determining [platelet] in cats.[37,42–44]
6. Optical flow cell methods (see Chapter 2 and Figure 2.5 for basic principles and analyzers)
   a. Light scatter (e.g., ADVIA analyzers): Low-angle (e.g., 2–3°) light scatter of individual cells is proportional to cell volume and can be used to help identify and size platelets, but single-angle light scatter cannot differentiate different types of particles that are of similar size (e.g., RBC fragments and platelets). ADVIA analyzers use the Mie theory of light scatter by homogeneous spheres to analyze RBCs and platelets.

(1) RBCs, which are internally homogeneous, are isovolumetrically sphered by the diluting fluid. Platelets tend to become spheroid after exposure to EDTA for 20–30 min and appear to be sufficiently homogeneous for most analyses.
(2) According to Mie scattering theory, the scattering of a fixed beam of light directed at homogeneous spheres is determined by cell volume and complex refractive index.[45] Refractive index is proportional to cell content and density, being greater in RBCs than in platelets; it can be assessed by the degree of high-angle (5–15°) light scatter. With amplification of platelet scatter signals, the combination of low-angle (volume) and high-angle (refractive index) scatter is used to form a volume/refractive index map that defines each cell and helps differentiate platelets from debris, RBC fragments, and RBC ghost cells. The number of 1–60 fL particles characterized as platelets in a given volume of fluid is the [platelet].
(3) This improves [platelet] accuracy over impedance or single-scatter optical methods, particularly in thrombocytopenic samples where the fraction of interfering particles is higher. However, some particles, including cell fragments, ghost RBCs, and certain lipid droplets, can scatter light similarly and be counted as platelets, thus falsely increasing the [platelet].

b. Fluorescence (e.g., Sysmex analyzers): A fluorescent dye stains nucleic acids in platelets and other cells, and fluorescence and light scatter properties are used to discriminate platelets. The Sysmex XN-V provides platelet concentrations by impedance, optical, and optical-fluorescence methods, using an oxazine-based dye for fluorescence.[39]

7. QBC (see Chapter 2 and Figure 2.6 for basic principles)
   a. The QBC detects the thickness of the cell layer above the lymphocyte and monocyte layer that has characteristic fluorescence of RNA or lipoprotein. The thickness of this layer (essentially a Pct) is converted to a [platelet] based on correlation studies with other analyzers, but the conversion formula is proprietary. Imprecision was high and analytical flags were common when used with feline samples.[25]
   b. Because the value is affected by platelet volume, it may not always match platelet concentrations based on other methods.[37]
   c. The analytical method is less affected by small platelet clumps than are impedance methods, but relatively mild false increases or decreases occur.[28]
8. Hemocytometer (see Chapter 2 and Figures 2.3 and 21.3 for basic principles)
   a. This manual technique is relatively labor intensive and has much lower precision than most automated methods, but it is adequate for most diagnostic uses and may be preferred when there are many giant platelets (greater than an analyzer's size threshold for platelets).
   b. Platelets are enumerated microscopically in defined grid regions after sample dilution and lysis of RBCs. As with any method, platelet clumping prevents accurate measurement of [platelet], but results may be more accurate than with automated methods when clumps are present.[25] Other platelet parameters are not assessed.

C. MPV
1. Generally, optical analyzers assess the forward or low-angle (e.g., 2–3°) light scatter generated by each particle identified as a platelet and transform the signal into a volume measure; greater detection of forward light scatter equals a larger platelet. Although affected mostly by cell volume, low-angle scatter is also affected by internal refractive index, which is primarily assessed by high-angle scatter, so evaluation at both scatter

angles, as is done by ADVIA analyzers, can provide a more accurate volume measurement.

2. Impedance methods convert the increase in impedance caused by each platelet passing through an aperture placed between two electrodes into a volume measure; greater impedance equals a larger platelet.
3. MPV can also be calculated as the quotient of the Pct divided by the platelet concentration and corrected for units (see relationship in Eq. 4.1a).
4. Platelets do not contribute to the MPV if they are larger than the upper threshold for platelets, either individually or because they are in clumps. More large platelets are excluded by impedance methods than by optical methods, but the diluting fluids used to sphere platelets in optical analyzers may falsely decrease the MPV.[46–48]
5. MPV is affected by anticoagulants, sample storage temperature, and sample storage time. These variables usually have relatively minor effects (< 20 % differences), but the effects should be recognized when values are interpreted, particularly for values near the upper or lower reference limits. Other platelet parameters such as Pct, PDW, and the proportion of large platelets may also be affected.
   a. Anticoagulant: EDTA is routinely used. Although platelets undergo mild activation changes in EDTA samples,[49] one of the changes is to become more spheroid; this is an advantage for ADVIA analysis in that the ADVIA MPV method assumes spherical cells and the ADVIA's buffer spheres RBCs but not platelets. Although EDTA promotes spheroid change, platelets may not be spheres when tested, leading to inaccuracies in MPV.[26] This depends in part on exposure time. Compared to EDTA, citrate better maintains platelets in their discoid shape (they sphere less and more slowly in citrated blood), thus yielding falsely increased MPV values with the ADVIA method.[26,31]
   b. Storage time: With impedance measurements and EDTA-anticoagulated samples, time-dependent artifactual increases occurred within 5 min (these are usually not recognized because of delays to testing) and could result in 3-h values that were 150 % of baseline.[46,50] In one study, increases were about 15 % at 24 h compared to results generated within 2 h of blood collection.[38] With optical measurements in an ADVIA analyzer, values tended to be increased at 24 h and more so at 48 h (room temperature storage), but MPV decreased mildly in some samples.[37] In other studies, both increases and decreases have been reported.[51,52]
   c. Storage temperature: Increases in MPV are reported with room temperature or refrigerated storage,[53] but changes may be slower at 4 °C.[46]

D. PDW
   1. For ADVIA analyzers, PDW is calculated as the standard deviation of the distribution of platelet volumes and reported as a percentage (%) of the MPV, i.e., 100 × (standard deviation of MPV)/MPV.
   2. For some analyzers, the calculation excludes a certain proportion of the largest and smallest platelets. For example, considering the peak of the volume distribution curve to be 100 %, the difference (in fL) between the high and low platelet volumes at 20 % of the peak height is the PDW with some Sysmex analyzers.
   3. If large platelets are excluded from assessment or if platelets are clumped, the PDW is not reliable.

E. Pct
   1. Pcts are not measured like a spun Hct but are calculated from the MPV and [platelet]. Therefore, abnormalities or errors with either of these measurements (commonly from platelet clumping) affect the Pct (see Eq. 4.1).

Pct = MPV × [platelet]
With a MPV of 10 fL and a [platelet] of 300,000/μL, (**4.1a**)
the Pct = 10 fL × 300,000/μL = 3,000,000 fL/μL = 0.003 μL/μL = 0.3%

If [platelet] is reported in units of × 10^3/μL or × 10^9/L, e.g., 300 × 10^9/L, then
Pct (%) = MPV (fL) × [platelet]/10,000 (**4.1b**)
Example: Pct = 10 × 300/10,000 = 3000/10,000 = 0.3%

2. The QBC VetAutoread generates a platelet concentration based on a measured Pct, but instead of reporting the Pct, the layer thickness is converted by a proprietary formula to a platelet concentration.
3. In one study using an ADVIA, Pct tended to decrease at 24 h and more so at 48 h with room temperature sample storage, but some values increased mildly.[37]

F. MPC and PCDW (ADVIA)
1. Because MPC is based on light scatter and the assumption of cell sphericity (or near sphericity), if cells are not adequately sphered, MPC is falsely decreased. This may occur with some EDTA samples, particularly if analyzed soon after collection, and with citrated samples.[26] Decreased MPC with increased MPV may reflect platelet activation or insufficient sphering.
2. MPC is calculated from the mean refractive index of cells identified as platelets: MPC (g/dL) = (mean refractive index – 1.333) / 0.0018 dL/g, where 1.333 is the refractive index of H_2O, and 0.0018 is considered the average increment in refractive index caused by each g/dL of the typical proportions of platelet constituents including proteins, lipids, and carbohydrates.[45,50,54] Therefore, the refractive index of each platelet yields an estimate of the g/dL of that platelet's components that are not H_2O (its platelet component).
A refractive index histogram comprises results for individual platelets, the mean of which reflects the mean concentration of platelet cytoplasmic components (MPC). This is similar to CHCM being the mean concentration of RBC Hgb.
3. Increases and decreases in MPC have been shown to directly correlate with platelet density based on density gradient separation by centrifugation.[45]
4. PCDW and visual inspection of the platelet volume/refractive index scatterplot can be used to assess adequacy of platelet sphering; sphering may be insufficient if PCDW > 5.5 g/dL and platelets are diffusely scattered.[26,55]
5. MPC is also used to calculate the so-called MPM, which approximates the dry-weight mass of the average platelet in the sample. MPM (pg) is the product of MPC (g/dL) and MPV (fL) divided by 100 to correct for volume units. MPM is akin to MCH, the mean mass (pg) of Hgb per RBC. As with MCH, swelling or shrinkage of cells should have limited impact on the total dry-weight mass of each cell.[56] This mass is thought to reflect the functional potential of platelets, because dry mass correlates with granule content.

MICROSCOPIC FEATURES OF PLATELETS

I. General
A. In good smears of optimally collected and processed blood, platelets are present individually, with only a few touching one another unless there is a marked thrombocytosis. However, there are often platelet clumps of from 2–3 platelets to > 100 platelets (Figure 4.4).

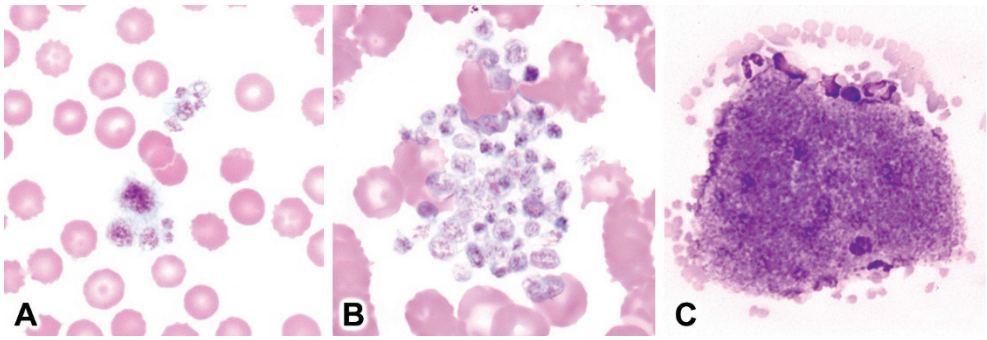


Figure 4.4. Small (A) and intermediate-sized (B) platelet clumps in the bodies of blood films, dogs. C. Large platelet clump in the feathered edge of a blood film, lower magnification, cow.

B. Clumps usually result from in vitro platelet activation (traumatic venipuncture or delayed contact with EDTA), but anticoagulant-induced agglutination of platelets, which is common in human samples, has been reported and may be present in as many as 5 % of equine samples.[27,57–59] Clumps are frequent in feline and bovine samples, presumably related in part to activation during collection. They may be found throughout a blood film but are readily found at its feathered edge, sides, and base. In people, EDTA-induced platelet clumps are typically of small to moderate size, numerous, relatively uniform, and contain platelets lacking features of activation. Descriptions of such clumps are rare in domestic mammals, but they were described as large in one case, and evidence was provided to suggest that mechanisms may differ in horses and people.[57]

II. Features
   A. Shape
      1. Nonactivated platelets are discoid and appear mostly round, oval, or elongate in blood films made from nonanticoagulated blood (Figure 4.5A).
      2. Platelets become spheroid with EDTA anticoagulation (also with citrate, but to a lesser degree) and more commonly appear round.
      3. Elongate platelets, which probably represent megakaryocyte pseudopod fragments (*proplatelets*), may be more numerous during stimulated thrombopoiesis (Figure 4.1B). Elongate platelets consistent with proplatelets have also been reported in Akitas with macrothrombocytopenia of undefined cause,[60] and mildly to moderately elongated platelets were present in a ragdoll cat that was homozygous for a particular mutation in the β1-tubulin gene; the cat may have been mildly thrombocytopenic based on blood smear evaluation.[43]
      4. Activated platelets may have peripheral pseudopodia and lack granules (Figure 4.5 B–D).
   B. Size
      1. In most healthy individuals, platelets are fairly uniform in size. However, cats have a greater PDW than other domestic mammals, and the diameter of some feline platelets may exceed that of feline RBCs.[61] This size variability may relate to a difference in platelet microtubule β1-tubulin protein in felids compared to other mammals.[43]
      2. There is not a universal definition of a giant platelet (large platelet, shift platelet, stress platelet, megaplatelet, megathrombocyte, or macrothrombocyte) or standard for how

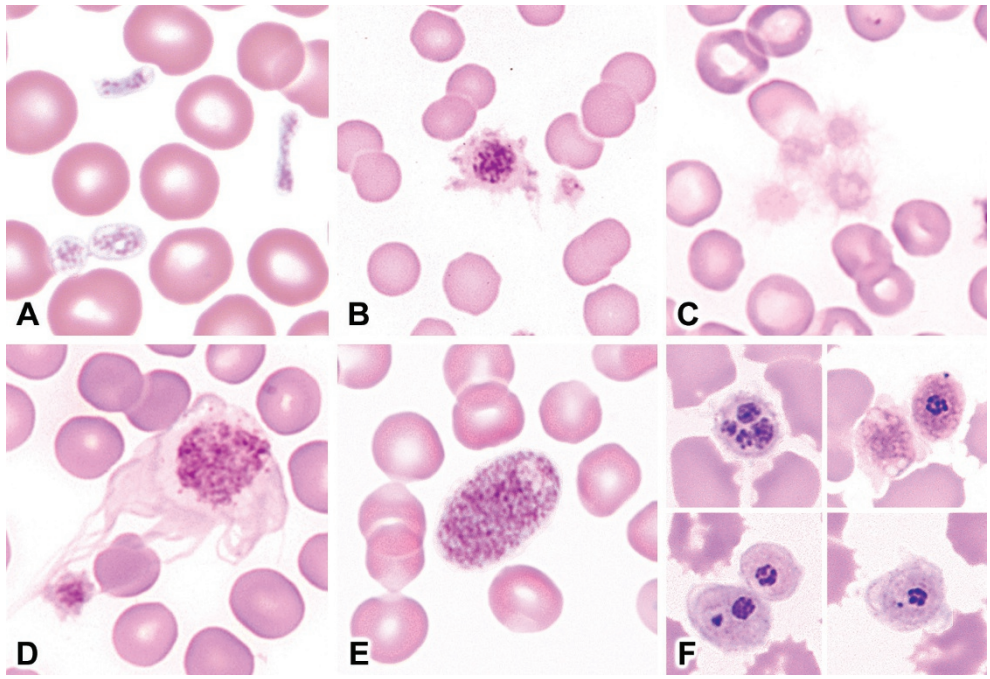


Figure 4.5. A. Oval and elongated platelets, fresh citrated blood, dog (most platelets were round in the same blood collected into EDTA). B. Activated platelet with pseudopods and centralized granules, cat. C. Cluster of four activated, degranulated platelets, cat. D. Giant platelet with pseudopods and centralized granules, Cavalier King Charles spaniel. E. Unactivated giant platelet, Cavalier King Charles spaniel. F. *Anaplasma platys* morulae in platelets, dog (slide courtesy of Dr. C. Lucidi, Universidade Estadual Paulista, Botucatu, Brazil).

many must be seen to report them. However, platelets that appear larger than normal RBCs are typically classified as *giant platelets*, and when the number of such platelets appears increased to the microscopist, they may be reported.

3. Because MPV is an average for all platelets, subpopulations of large platelets may be present without an increased MPV. Also, large platelets seen on a slide may not be included in MPV calculations from an instrument if they are too large to be detected as platelets.
4. On some blood films, many platelets appear larger than normal but not larger than RBCs. In such cases, giant platelets may not be reported (depends on laboratory protocol) but the MPV may be increased. Photographs that illustrate this can be found in a report of canine Bernard–Soulier syndrome, a rare congenital platelet defect.[62]
5. Giant platelets usually suggest new or ongoing stimulation of thrombopoiesis within a few days before sample collection, usually because of a destructive or consumptive thrombocytopenia. They may be accompanied by an increased MPV. However, they do not confirm adequate numbers of megakaryocytes, because thrombopoiesis may be stimulated when there is megakaryocyte hypoplasia.
6. Giant platelets also occur with certain congenital platelet disorders (e.g., May–Hegglin anomaly[63] or β1-tubulin defects[64] as shown in Figure 4.5D and E), clonal hemic disorders, splenic contraction, and some acute feline leukemia virus infections.

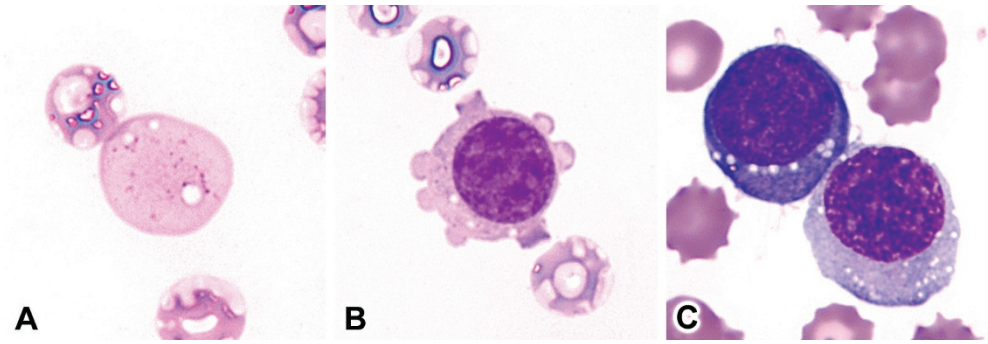


Figure 4.6. Abnormal giant, hypogranular platelet (A) and atypical megakaryoblast with cytoplasmic blebs (B), acute megakaryoblastic leukemia, dog (from ASVCP slide contributed by Dr. J. Messick et al., 1989); RBCs have refractile artifact. C. Megakaryoblasts, acute megakaryoblastic leukemia, dog (from ASVCP slide contributed by Dr. M. Ameri et al., 2006).

C. Inclusions
   1. Granules
      a. With Wright staining, a nonactivated platelet has clear to pale blue cytoplasm containing small pink to purple granules. These granules contain numerous substances that can be released to promote platelet functions, including hemostasis and tissue repair.
      b. With activation, the granules may be centralized (Figure 4.5B and D) or absent (secreted) (Figure 4.5C). Granules tend to be most prominent in feline and bovine platelets and least prominent in equine platelets.
      c. Abnormalities in granule size, shape, and staining may be seen with dysthrombopoiesis (Figure 4.6A). Small to large hypogranulated platelets may be prominent. Megakaryocytes may also be atypical (Figure 4.6B and C).
   2. *Anaplasma platys* (basonym *Ehrlichia platys*) morulae may be present in platelets (Figure 4.5F). They must be differentiated from stain precipitate and fragments of nuclear material. When they are suspected, specific analysis by polymerase chain reaction (PCR) may be used for confirmation.

THROMBOCYTOPENIA

I. General concepts
   A. *Thrombocytopenia* is a pathologic state in which a patient's [platelet] is less than a valid lower reference limit. A decreased sample [platelet] does not mean a patient is thrombocytopenic unless pseudothrombocytopenia is excluded. Platelet clumping must be absent or mild on blood smear evaluation to trust automated platelet concentrations.
   B. Care must be taken to consider an animal's signalment and to either use appropriate RIs or adjust interpretation according to expected deviations from general RIs. Comparison to the individual's historical or baseline values may be helpful.
      1. Breed: Many greyhounds[65] have substantially lower platelet concentrations than dogs of other breeds, and there is evidence of other canine breed variations with as much as 150,000–200,000/μL differences between median platelet concentrations for breeds

with lower concentrations (German shepherds, Labrador retrievers) and breeds with higher concentrations (Pekingese, pug, Pomeranian).[66] Shiba inus,[67] dogues de Bordeaux,[68] Polish ogar dogs,[69] and some nongreyhound sighthounds may also have substantially lower platelet concentrations than other dogs. Conversely, the lower reference limit for [platelet] in Icelandic horses in Austria was reportedly greater than that for most reference populations.[41]

2. Sex: In dogs, [platelet] appears to be mildly greater (up to 10 %) in females than in males, and in intact females or males than in their neutered counterparts.[66,70]
3. Age: In dogs, [platelet] may gradually increase by a total of about 100,000/μL as dogs age from 1–2 yr to 12 yr.[66] Values in neonates may be different from adults, e.g., greater in Holstein calves than in adult Holstein cows.[71]

C. The major clinical significance of thrombocytopenia is the potentiation of bleeding when platelet concentrations are markedly decreased (usually < 30,000/μL). Petechiae and ecchymoses are the hallmarks of severe thrombocytopenia, though severe thrombocytopenia (e.g., 3,000/μL) can occur without hemorrhage. Mucosal bleeding (epistaxis, hematochezia, or melena), hematuria, hyphema, and prolonged hemorrhage after accidental or intentional trauma (e.g., venipuncture) are common. Hematomas unrelated to venipuncture are more likely to form in cattle or horses than in dogs and cats. In one study,[72] hemoperitoneum was reportedly the most common clinical sign in thrombocytopenic dogs, but hemoperitoneum was not defined. Neurologic signs could reflect bleeding in the central nervous system.

D. Thrombocytopenia is not a disease. Its presence reflects one of many potential underlying disorders[72–76] caused by one or more of only several pathogenic mechanisms (Figure 4.2).

II. Disorders and conditions (Table 4.2)

A. *Pseudothrombocytopenia*, false thrombocytopenia that occurs when not all the platelets in a sample are counted, is very common and must be excluded prior to interpreting a

Table 4.2. Disorders and conditions that cause thrombocytopenia

Decreased or defective platelet production (may involve multiple hemic cell lines)
Asymptomatic macrothrombocytopenia caused by a tubulin defect (Cavalier King Charles spaniels,* Norfolk terriers, Cairn terriers, sporadic other dogs, and a ragdoll cat) or May–Hegglin anomaly (may be symptomatic)
Bernard–Soulier syndrome (Cocker spaniels)
Acquired amegakaryocytic thrombocytopenia
Bovine neonatal pancytopenia
Drugs/toxicants
Predictable: chemotherapeutic agents,* estrogens in dogs (exogenous, endogenous), bracken fern poisoning (ruminants), trichothecene mycotoxins
Idiosyncratic: phenylbutazone, meclofenamic acid, trimethoprim-sulfamethoxazole, griseofulvin, albendazole
Infectious* (usually multifactorial; see the text)
Irradiation (whole body or extensive): prior to autologous marrow transplantation
Marrow replacement:* bone marrow neoplasms (primary hemic or metastatic), myelofibrosis, osteopetrosis

Table 4.2. Disorders and conditions that cause thrombocytopenia (Continued)
Megakaryoblastic leukemia
Myelonecrosis: infections, neoplasms, toxicants
Decreased pulmonary thrombopoiesis (possibly): right-to-left shunting, severe pulmonary disease
Decreased platelet survival (accelerated platelet destruction and consumption)
Immunologic
Primary (idiopathic) IMT*
Secondary IMT
Drug induced: gold salts, sulfonamides
Infectious (usually multifactorial; see the text)*
Neonatal alloimmune thrombocytopenia
Neoplasms (usually multifactorial; see the text)*
Posttransfusion purpura
Systemic immune-mediated disease: SLE, Evans syndrome
Nonimmunologic
Platelet activation with accelerated consumption or utilization
Localized intravascular coagulation: hemangiosarcoma, hemorrhage, thrombosis
DIC:* envenomation, hepatic disease, infections, massive necrosis, pancreatitis, neoplasms, overheating, septicemia
Drugs: protamine sulfate, BH3 mimetics (proapoptotic)
Envenomation (without DIC)
Vasculitis/endocarditis:* Rocky Mountain spotted fever, canine herpesvirus infection, dirofilariasis, angiostrongylosis, bacterial endocarditis, hemolytic uremic syndrome, cutaneous and renal glomerular vasculopathy
Blood loss, acute and severe: anticoagulant rodenticides
Abnormal platelet distribution (sequestration): splenomegaly, severe hypothermia
Hemodilution: massive infusion with colloids, crystalloids, platelet-poor blood products
Idiopathic mechanism or multifactorial (decreased production and decreased survival)
Infections:* *Anaplasma platys*, *Anaplasma phagocytophilum*, *Babesia canis*, *Babesia gibsoni*, *Borrelia* spp., bovine viral diarrhea virus, canine distemper virus, canine parvovirus, *Cytauxzoon felis*, *Ehrlichia* spp., equine infectious anemia virus, feline leukemia virus, feline immunodeficiency virus, *Histoplasma capsulatum*, *Leishmania* spp., *Leptospira* spp., severe fever with thrombocytopenia syndrome, *Theileria* spp., and *Trypanosoma* spp.
Endotoxemia
Possibly hepatic failure
Neoplasms:* carcinomas, hemangiosarcoma, histiocytic sarcoma, other sarcomas, lymphoma, leukemias
Drugs/toxicants: *Cuscuta capestris* (alfalfa dodder)
Hypophosphatemia associated with hyperalimentation
Anaphylaxis

* A relatively common disorder, condition, or mechanism

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Platelet concentrations in greyhounds and some other breeds (see the text) may be substantially lower than for most breeds of dogs. Thrombocytopenia should be confirmed by blood smear review. Pseudothrombocytopenia caused by platelet clumping or giant platelets should be considered before pursuing causes of true thrombocytopenia.

[platelet]. It can be suspected when automated analyzers indicate the presence of platelet clumps (but also occurs without being flagged)[25,26] or when platelet clumps or many giant platelets are present in blood smears.

1. Pseudothrombocytopenia often occurs because blood collection causes platelet activation and aggregation. Therefore, platelets are clumped and not counted individually. This may occur with traumatic venipuncture (e.g., probing with the needle) or delayed contact with EDTA (slow draw into a syringe before transfer to tube, poor sample mixing) whereby coagulation is activated (e.g., by tissue factor exposure), thrombin is generated, and thrombin activates platelets. Blood collection for accurate platelet assessment requires the same care as recommended for coagulation testing (see Chapter 5).
2. Pseudothrombocytopenia may occur with automated analyzers, particularly impedance analyzers, when many large platelets are present (e.g., in dogs or cats with asymptomatic macrothrombocytopenia). For such samples, platelet concentrations should be determined by microscopic methods.
3. Pseudothrombocytopenia can be induced by EDTA anticoagulation, although this has been reported rarely in domestic mammals.[27,57–59] In people, EDTA unmasks nonpathologic antigenic sites on platelet membranes so that immunoglobulins, often cold agglutinins, bind and bridge platelets together in vitro.[77] An EDTA effect is supported by measuring substantially greater platelet concentrations in citrated or heparinized samples from the animal, but mechanisms may be different in domestic mammals.[57] Based on higher platelet concentrations in citrate samples than in paired EDTA samples, EDTA-induced pseudothrombocytopenia was suspected in 5 of 120 (4.2 %) healthy horses and 2 of 20 (10 %) horses with systemic inflammatory disease syndrome, findings similar to another study,[59] but microscopic assessment for clumping was not reported, and there were no differences in analyzer-reported large platelets or platelet clumps between sample types.[78]
4. In people, cold agglutinins rarely have been reported to clump platelets independent of a particular anticoagulant (not EDTA-dependent); assessment at 37 °C can solubilize cold agglutinins and eliminate the clumping.

B. Decreased or defective platelet production

1. Adequate platelet production requires a healthy megakaryocyte population; there must be enough megakaryocytes and they must be shedding platelets. Megakaryocytes can be unhealthy because of generalized bone marrow disease or because of megakaryocyte-specific disorders that either diminish megakaryopoiesis or impair thrombopoiesis. Unexplained bicytopenia and pancytopenia suggest the possibility of generalized bone marrow disease.
2. Asymptomatic macrothrombocytopenia is a common inherited disorder in Cavalier King Charles spaniels and has been documented in other breeds of dog.[64,79–81] Mutations in the gene for β1-tubulin, a protein involved in the assembly of microtubules, are associated with defective thrombopoiesis and production of fewer but generally larger platelets (*macrothrombocytes*), yielding a Pct that is usually similar to that of unaffected dogs. Platelet concentrations of 50–100 × 10^3/μL are common, and they may be < 25,000/μL in some dogs, but affected dogs have no clinical hemostatic disorder. The percentage of platelets that are large generally increases with decreasing [platelet] in affected dogs.
   a. Although initially reported to occur with an autosomal recessive inheritance pattern in Cavalier King Charles spaniels,[82] platelet concentrations of 100,000–200,000/μL

in dogs heterozygous for the point mutation suggest that it may have an autosomal dominant inheritance.[64] When assessed, these platelets from homozygotes have lacked well-developed circumferential microtubular rings.[81]

b. A single nucleotide polymorphism identified in some Norfolk terriers and some Cairn terriers predicts a β1-tubulin alteration that is different from the defect in Cavalier King Charles spaniels but associated with a similar platelet phenotype in homozygotes.[64] This defect may have an autosomal recessive inheritance pattern.

c. One asymptomatic ragdoll cat homozygous for a different β1-tubulin gene mutation appeared to be mildly thrombocytopenic (blood smear estimate) and had large, elongated platelets.[43]

d. Asymptomatic macrothrombocytopenia has been reported in a pug with May–Hegglin anomaly caused by a mutation in the gene for myosin 9.[83] May–Hegglin anomaly may be symptomatic or asymptomatic in people; this is likely true for other species.

e. Recognition of asymptomatic macrothrombocytopenia is important to prevent unnecessary therapy and to employ appropriate methods for accurate platelet concentrations.

(1) Microscopic hemocytometer counts or estimates from blood smears are useful.

(2) Automated impedance methods are unreliable when a substantial number of platelets are larger than the analyzer's upper threshold (which varies with the analyzer); results for [platelet] will be falsely low, if provided.

(3) Optical analysis improves detection of platelets because internal cell characteristics are assessed in addition to volume. However, [platelet] may be falsely low, particularly with more severe thrombocytopenia and more large platelets.[37]

(4) The QBC VetAutoread provides falsely increased values for [platelet] in dogs with macrothrombocytopenia, but the reported values may reasonably reflect the Pct and therefore overall functional hemostatic potential.

3. Bernard–Soulier syndrome is a rare inherited platelet disorder reported in Cocker spaniels of European origin.[62] Affected dogs have mild to moderate thrombocytopenia, an increased MPV, and defective primary hemostasis that can cause clinical bleeding problems. In people with Bernard–Soulier syndrome, the decrease in platelet glycoprotein Ib decreases stimulation of hepatic Tpo production, thus decreasing thrombopoiesis.[84]

4. Acquired selective amegakaryocytic thrombocytopenia is rarely reported in dogs and cats.[85,86] Bone marrow aspirate and core samples must be of excellent quality to conclude reliably that megakaryocytes are absent.

a. Some cases may be immune mediated, as in people with documented antibody-mediated or cell-mediated[87] destruction of megakaryocytes or their precursors, or with antibody-mediated destruction of required cytokines.[88,89]

b. Antimegakaryocyte antibodies can impair thrombopoiesis[90,91] without megakaryocytic hypoplasia or aplasia. Dogs injected with rabbit anti–canine platelet antiserum developed marked thrombocytopenia with morphologic changes in megakaryocytes that suggested the possibility of impaired megakaryocyte function without megakaryocytic hypoplasia.[92] Plasma from some people with IMT inhibits megakaryopoiesis and thrombopoiesis in cell cultures.

5. Bovine neonatal pancytopenia was the name given to a disorder of calves (usually 1–4 wk of age) in which cytopenias are mediated by post-vaccination colostral alloantibodies

that target major histocompatibility class I antigens on a calf's bone marrow precursors and circulating WBCs and platelets.[93] Major clinical and laboratory findings include severe post-colostral leukopenia and thrombocytopenia, commonly hemorrhage, and the development of generalized bone marrow hypoplasia. A European outbreak began in 2007 in association with a particular BVD vaccine (and adjuvant) that contained residual bovine proteins from the use of bovine renal cells to culture the BVD virus used in the vaccine. Dams that generated alloantibodies to these proteins passed them via colostrum to calves, some of which were affected. That vaccine was discontinued, but similar cases of alloimmune pancytopenia have since been reported in calves.[94] The cause is unknown, but other vaccines produced with allogeneic cells could potentially cause this disorder.

6. Drugs/toxicants
   a. Drugs with predictable and dose-dependent myelosuppressive effects (examples)
      (1) Antineoplastic chemotherapeutic agents, including alkylating agents, antimetabolites, anthracyclines, and antibiotics (e.g., doxorubicin), frequently induce thrombocytopenia 1–2 wk after appropriate or excessive (overdose) drug administration.[95–97]
      (2) Estrogens from exogenous sources[98] or endogenous sources (e.g., testicular Sertoli cell neoplasm,[99] rare ovarian granulosa cell neoplasms,[100] and rare interstitial cell neoplasms[101] in dogs) can induce thrombocytopenia or aplastic pancytopenia. Suppressive doses cause thrombocytopenia after a week or more, and thrombocytopenia may persist for weeks. Myelosuppression may be mediated by an inhibitor produced by thymic stromal cells in response to estrogen.[102]
      (3) Bracken fern poisoning may induce aplastic anemia with thrombocytopenia in ruminants,[103] as may trichothecene mycotoxins.[104] Trichothecene mycotoxins in cat food were the suspected cause of a feline outbreak of myelosuppressive pancytopenia (with thrombocytopenia) in the United Kingdom.[105]
   b. Drugs (toxicants) with idiosyncratic (i.e., sporadic and unpredictable) myelosuppressive effects (examples)
      (1) Idiosyncratic myelosuppression is suspected much more often than it is proven; reported associations may not reflect causation. Relapse of thrombocytopenia with rechallenge after initial recovery would support a cause-and-effect relationship, but such rechallenges are avoided. When suspected, potential offending drugs are withdrawn, but subsequent improvement may or may not be due to drug withdrawal.
      (2) Phenylbutazone and meclofenamic acid are nonsteroidal anti-inflammatory drugs that have been associated with bone marrow hypoplasia and bicytopenia or pancytopenia in dogs and, for phenylbutazone, probably in horses.[106–108]
      (3) Trimethoprim-sulfadiazine and trimethoprim-sulfonamide have been associated with aplastic anemia (aplastic pancytopenia) in dogs and cats.[109–111] This occurrence is uncommon and unpredictable at doses commonly used. However, potentiated sulfonamides predictably inhibit sequential steps in folate pathways, thus suppressing DNA synthesis. This may contribute to aplastic anemia and thrombocytopenia. These drugs may also cause immune-mediated platelet destruction via trimethoprim-sulfa–dependent antibodies.

(4) Griseofulvin has caused marrow suppression in cats,[112,113] and an idiosyncratic reaction to albendazole has been implicated in a cat.[114]
(5) Albendazole[114] and fenbendazole[108,115,116] have been implicated in dogs, and albendazole myelosuppression has been reported in people.[117]

7. Infections: The pathogenesis of thrombocytopenia associated with many infections is multifactorial (see Thrombocytopenia, Section II.F.1) but often includes decreased platelet production. Several mechanisms of decreased production may be involved.
   a. Direct infection of megakaryocytes or other hematopoietic precursors may decrease megakaryopoiesis and therefore thrombopoiesis or decrease thrombopoiesis despite adequate megakaryopoiesis. Potential examples are infections with acute bovine viral diarrhea virus[118] and canine distemper virus.[119]
   b. Myelosuppressive cytokines may be produced in response to infection (e.g., with equine infectious anemia virus).[120,121]
   c. Some infections (e.g., chronic ehrlichiosis caused by *Ehrlichia canis* and canine parvoviral infection) are associated with acute (parvovirus) or chronic (myelosuppressive ehrlichiosis)[122] generalized bone marrow hypoplasia and thrombocytopenia, but the mechanisms are not clear. Canine parvoviral infections may cause suppression thrombocytopenia by direct infection of hematopoietic precursor cells. However, viral antigen has minimally and inconsistently been found in the bone marrow of experimentally infected dogs and, when present, was not in recognizable cells of the megakaryocyte series.[123,124] Thrombocytopenia in parvovirus may also be caused by septicemia and/or endotoxemia associated with severe enteritis.
   d. Production failure may contribute to thrombocytopenia occurring in some cats with feline leukemia virus infections, but the effects may be indirect via secondary disorders, including hemic neoplasms.
8. Irradiation (whole body or extensive): This can cause generalized marrow suppression and thrombocytopenia through widespread cell death. Thrombocytopenia was prolonged (weeks to months) and severe in dogs receiving autologous bone marrow transplants after marrow-ablative total body irradiation.[125,126]
9. Marrow replacement (myelophthisis): Bone marrow neoplasms (primary hemic or metastatic), myelofibrosis, osteopetrosis, or osteosclerosis may each induce myelosuppression. Mechanisms may include physical replacement of normal cell populations, competition for nutrients, obstruction of blood supply, lysis of marrow cells, and secretion of inhibitors. Megakaryocytic leukemia may be associated with thrombocytopenia,[127–129] probably because of dysthrombopoiesis and decreased thrombopoiesis.
10. Megakaryoblastic leukemia: Although there can be increased thrombopoiesis and thrombocytosis, thrombocytopenia from decreased thrombopoiesis may occur because of megakaryocyte defects or immaturity.
11. Myelonecrosis (with or without myelofibrosis): Infections, neoplasms, or toxicants may cause enough marrow necrosis to contribute to thrombocytopenia.[130,131]
12. Decreased pulmonary production of platelets because of a decrease in megakaryocytes reaching the lungs (e.g., right-to-left shunting, cardiopulmonary bypass surgery) or because of pulmonary disease (e.g., severe acute respiratory response syndrome) have been proposed as potential contributors to thrombocytopenia in people.[5]

13. Hepatic failure: Although thrombocytopenia is not well documented in hepatic failure of domestic mammals, decreased hepatic Tpo production is thought to contribute to thrombocytopenia in human patients with hepatic failure.[132,133]

C. Decreased platelet survival (accelerated platelet destruction or consumption)
   1. If increased platelet production does not compensate for accelerated platelet destruction or consumption, thrombocytopenia ensues.
      a. Platelet *consumption* usually refers to the use of platelets during hemostatic functions, whether physiologic or pathologic.
      b. Platelet *destruction* refers to the death of platelets by other means, often immune-mediated destruction. However, increased destruction may be nonimmune:[20,21,134,135]
         (1) Increased platelet apoptosis and clearance may occur with myelodysplastic syndromes, certain infections, apoptosis-inducing drugs, or in conditions that decrease the activity of platelet protein kinase A, an important regulator of platelet apoptosis. (It may also occur secondary to cytotoxic T lymphocytes in IMT.)[136]
         (2) Increased hepatic platelet clearance can occur via asialoglycoprotein receptors when glycosylation of platelet surface proteins is decreased, e.g., by neuraminidases released from certain bacteria or with the binding of von Willebrand factor. (Some antiplatelet antibodies may also promote desialylation.)
   2. Immunologic causes of decreased platelet survival
      a. General concepts
         (1) IMT can be caused by a variety of acquired disorders with the commonality that thrombocytopenia is mediated largely by the immune system. In most cases, an animal's immune system produces antibodies that bind directly (e.g., autoantibody, alloantibody) or indirectly (e.g., to an adsorbed antigen via immune adherence) to its own platelets (PSAIg). This leads to accelerated platelet destruction by the MPS, particularly in the spleen. Destruction is mediated largely by Fc receptors for PSAIgG, but also by complement receptors for platelet surface-associated complement and potentially to some degree by asialoglycoprotein (Ashwell–Morell) receptors or receptors recognizing apoptotic surface changes after T lymphocyte- or antibody-induced platelet changes.[21,137] Additionally, platelets may be lysed directly by the complement membrane attack complex or by autoreactive cytotoxic T lymphocytes, and there may be immunologic suppression of platelet production by the effects of autoantibodies or autoreactive T lymphocytes on megakaryocytes.[137] The importance of each mechanism may vary with the individual.[136]
         (2) IMT caused by colostral alloantibodies directed toward alloantigens is alloimmune thrombocytopenia, not autoimmune thrombocytopenia. Alloantibodies also mediate platelet destruction after incompatible platelet transfusions.[138]
         (3) IMT not associated with a detected disease or condition is considered *primary IMT (primary ITP)* and presumed to be autoimmune, although undetected underlying disease does not exclude underlying disease. Although clearly immune mediated, primary IMT is idiopathic in that the underlying triggers are unknown. Whatever the triggers, there is a loss of self-tolerance and platelet/megakaryocyte antigens are attacked.[137]

(4) IMT that is part of a broader autoimmune disease (e.g., SLE, Evans syndrome) is considered *secondary IMT (secondary ITP)*, as is autoimmune thrombocytopenia associated with underlying infections, neoplasms, or recognized immunodeficiency disorders.[136,137] However, underlying triggers for what is currently considered primary IMT might involve infections or chemical exposures that have not been recognized.[137]

(5) IMT mediated by immune adherence of immune complexes to platelets or by antibodies to adsorbed non–self-antigens would technically not be autoimmune or alloimmune, because neither autoantibodies nor alloantibodies would be involved. However, the net effect is the same.

(6) The abbreviation "ITP" is commonly used for primary and secondary autoimmune thrombocytopenia. It originally was used for "idiopathic thrombocytopenic purpura" in human patients, but it came to be used for "immune thrombocytopenic purpura" once an immunologic mechanism was demonstrated. However, purpura is often not present in patients with IMT, so the current recommendation in human medicine is to continue the long-term use of ITP, but with the understanding that it is an abbreviation for "immune thrombocytopenia."[139] Immune-mediated thrombocytopenia (IMT) is used in this text, consistent with IMHA and IMN (immune-mediated neutropenia).

b. Primary IMT

(1) Presumed primary IMT is well documented and relatively common only in dogs and people but appears to occur in cats and horses as well.[140–144] At least in people, it is a disorder of both decreased platelet survival and impaired platelet production.[145] Platelet production is inadequate to balance the increased rate of platelet destruction, despite adequate to increased megakaryocytes in most cases.

(a) Decreased platelet survival: Immunoblotting and immunoprecipitation studies have confirmed a role for platelet membrane antigen targets in at least some cases of canine idiopathic IMT.[146,147] In people, most antibodies are directed against autoepitopes on the most abundant platelet membrane proteins, $\alpha_2\beta_3$ (the fibrinogen receptor) and glycoprotein Ib (von Willebrand factor receptor). Platelets may also be destroyed via cytotoxic T lymphocytes.

(b) Decreased platelet production: Evidence supports that some antibodies target platelet epitopes on megakaryocytes and impair proplatelet formation and thrombopoiesis. In addition, cytotoxic T lymphocytes may attack megakaryocytes and impair thrombopoiesis. Furthermore, increases in serum [Tpo] are mild in many human IMT patients, thus creating a role for Tpo receptor agonist therapy. Theories for a subdued Tpo response in IMT include a switch from Tpo-inducing platelet clearance by hepatic asialoglycoprotein receptors to primarily non–Tpo-inducing splenic platelet clearance via IgG and complement receptors.[9]

(2) Common laboratory features in dogs are thrombocytopenia (usually < $50 \times 10^3/\mu L$ and often < $10 \times 10^3/\mu L$), anemia (often associated with hemorrhage), megakaryocytic hyperplasia (megakaryocytic hypoplasia can occur), and a MPV that is increased, decreased, or WRI.[148,149] These findings are not specific for IMT. A diagnosis of IMT should not be based on the degree of thrombocytopenia alone because severity can be the same in other conditions.

(3) Primary IMT is diagnosed based on clinical findings and exclusion of other causes; apparent response to immunosuppressive therapy may also be supportive, but it may be only coincidental.[150] Bone marrow evaluation is usually not indicated or useful. There is little indication to test for antiplatelet antibodies (see Antibody Tests for Immune-mediated Thrombocytopenia at the end of this chapter).

c. Drug-induced IMT

(1) Exposure to certain drugs may lead to increased PSAIg and therefore accelerated platelet destruction because of the production of drug-dependent or drug-independent antibodies. Platelet production may also be impaired.[151]

(a) *Drug-independent antibodies* do not require the drug for binding; they may bind to a platelet epitope that is cross-reactive with the drug or a metabolite.

(b) *Drug-dependent antibodies* can specifically bind to any of the following: a drug or a metabolite on the platelet surface, a platelet surface epitope exposed by the presence of the drug or a metabolite, or a combined drug-platelet neoantigen on the platelet membrane. Immune complexes involving a drug may bind via platelet Fc receptors in species that have them (e.g., people) or by immune adherence in species with platelet complement receptors (likely dogs).[152]

(2) Drug-induced IMT is suspected when otherwise unexplained thrombocytopenia, usually severe, develops a few days (5–10) after drug exposure, and when the thrombocytopenia resolves rapidly after drug withdrawal. If re-exposure to the drug occurs, thrombocytopenia must recur to support drug-induced IMT.

(3) Strict criteria to be met for confirmation of drug-induced IMT by drug-dependent antibodies are the following:

(a) Increased PSAIg in a patient that developed thrombocytopenia at least a few days after drug exposure

(b) Patient plasma antibodies that bind in vitro to platelets or platelet antigens in the presence, but not in the absence, of drug or a metabolite (such assays and experience with such assays are very limited in veterinary medicine)

(c) Resolution of both thrombocytopenia and increased PSAIg after drug withdrawal

(d) Relapse after re-exposure to the drug (not recommended)

(4) Numerous drugs have been suspected to cause IMT in people and dogs, but cause-and-effect are clearer in people.[151,153] The following are the most common demonstrated causes of drug-induced IMT in people: trimethoprim/sulfamethoxazole, vancomycin, penicillin, rifampin, carbamazepine, ceftriaxone, ibuprofen, quinine, quinidine, mirtazapine, oxaliplatin, suramin, abciximab, tirofiban, eptifibatide, and heparin.

(5) In dogs, gold salts (auranofin, gold sodium thiomalate, and possibly aurothioglucose)[154] and sulfonamides (with or without trimethoprim)[155,156] have been strongly implicated, with support from platelet antibody assays. Methimazole and propylthiouracil have been incriminated in cats,[157,158] and penicillin and trimethoprim-sulfadoxine may have led to IMT in horses.[159]

(6) Heparin, a common cause of immune complex–induced IMT in people, has not been reported to produce an analogous condition in veterinary species. The pathogenesis of heparin-induced IMT involves platelet Fc receptors for

IgG that is bound to complexes of heparin and platelet factor 4. However, Fc receptors for IgG could not be demonstrated on rabbit platelets[160] and may not be present on platelets of other domestic mammals. No functional equivalent of the human FcγRII was found on canine platelets.[152]

d. IMT associated with infection (usually multifactorial; see Thrombocytopenia, Section II.F.1)
   (1) Pathogeneses of thrombocytopenia associated with infection are usually multifactorial or unknown, but many bacterial, viral, fungal, and protozoal diseases have been associated with IMT in people. Similar associations are likely in domestic mammals.
   (2) Infection-associated IMT may be caused by (i) cross-reactive antibodies, (ii) bacteria-induced production of autoantibodies, (iii) exposure of otherwise hidden platelet membrane antigens by the organism, (iv) binding of anti-organism antibodies to the infectious agent attached to the platelet membrane, or (v) induction of immune complexes that adhere to the platelet membrane.
   (3) Infection-associated IMT may occur with acute canine ehrlichiosis[161,162] and, by extension, infections with related organisms of the genera *Ehrlichia* and *Anaplasma*. Thrombocytopenia also occurs in cats with *E. canis* or *A. phagocytophilum* infections,[163] and in horses with *A. phagocytophilum* infections. Other infectious diseases with a likely immune component to the thrombocytopenia are Rocky Mountain spotted fever,[164] histoplasmosis,[165] leishmaniasis,[162,166,167] distemper or modified live virus distemper vaccination,[119] equine infectious anemia,[168] and babesiosis.[169]
   (4) Some people have considered vaccination a precipitating event for clinically manifested IMT in dogs, but supporting data are lacking.
e. Neonatal alloimmune thrombocytopenia
   (1) Thrombocytopenia occurs when passively acquired (mainly via colostrum) maternal alloantibodies to paternal epitopes on a neonate's platelets circulate in the neonate's blood and mediate platelet destruction. The maternal alloantibodies are produced when the dam is sufficiently exposed to fetal platelets possessing paternally derived antigenic determinants that are recognized as foreign. Specific alloantigens have not yet been identified, but assessment of equine and canine genes for major platelet membrane proteins have identified polymorphisms that may be associated with alloimmune disease.[170,171] Neonatal alloimmune thrombocytopenia has been described in horses, mule foals, and pigs.[172–174]
   (2) This disorder must be differentiated from other causes of thrombocytopenia in neonates, especially sepsis. Confirmation entails detection of increased PSAIg in the neonate but not in the dam, and demonstration of the presence of antibodies in the dam's blood that react with paternal and neonatal, but not maternal, platelets. Validated tests may be unavailable.
   (3) Thrombocytopenia concurrent with neutropenia and ulcerative dermatitis has been described in neonatal foals and may have an alloimmune pathogenesis.[175]
f. Neoplasms (usually multifactorial; see Thrombocytopenia, Section II.F.2)
   (1) Thrombocytopenia occurs commonly in animals with neoplasms. The cause is often multifactorial but may include immunologic mechanisms.

(2) Antibodies, possibly in the form of immune complexes, have been indirectly implicated in mediating thrombocytopenia in some dogs with a variety of hemic and nonhemic neoplasms.[176] IMT has been associated with lymphoma in dogs and horses.[177,178]

g. Posttransfusion purpura
   (1) In human transfusion recipients, this condition occurs about 1 wk after transfusion. It is associated with severe thrombocytopenia and high titers of platelet-reactive alloantibodies that mediate the destruction of the patient's own platelets in addition to the transfused platelets.
   (2) Rarely, posttransfusion thrombocytopenia has been reported in dogs, but the pathogeneses of thrombocytopenia in each case have not been established.[138,179]

h. Systemic immune-mediated diseases such as SLE are associated with polyclonal B-lymphocyte activation and the production of autoantibodies directed against multiple targets, one of which may be platelets.
   (1) In dogs with SLE, there is evidence, though rarely documented, of increased PSAIg.[148]
   (2) Evans syndrome
      (a) This was first described in people as idiopathic immune hemolytic anemia and concurrent thrombocytopenia or neutropenia,[180] but the term is now used to refer to concurrent or sequential IMT and IMHA. In at least some human patients, the antibodies mediating anemia and thrombocytopenia are distinct.[181] Information is unavailable for animals.
      (b) IMHA with concurrent presumed IMT occurs in dogs (first reported in 1965[182]).[183] An immune pathogenesis has been implicated in some cases by indirect assays for platelet-reactive or megakaryocyte-reactive antibodies in patient plasma.[148] However, dogs with IMHA are often prothrombotic, so thrombocytopenia may reflect platelet consumption instead of, or in addition to, immunologic destruction.[184]
      (c) Evans syndrome secondary to lymphoma has been suspected in horses.[185]

3. Nonimmunologic causes of decreased platelet survival
   a. Platelet activation with accelerated consumption or use: Any disorder associated with increased platelet activation may accelerate platelet consumption and lead to thrombocytopenia if platelet production does not compensate enough.
      (1) Localized (controlled) intravascular coagulation (e.g., with hemangiosarcoma, thrombosis, or hemorrhage) may be associated with platelet consumption at the site(s) where coagulation occurs.
         (a) Activation of the coagulation cascade leads to the generation of thrombin (Factor IIa), a potent platelet activator.
         (b) Thrombin activates platelets. Activated platelets release platelet-activating substances that recruit and consume more platelets locally. Thrombocytopenia may ensue if the lesion is severe enough.[186]
      (2) DIC: Widespread, uncontrolled activation of the coagulation cascade may produce thrombocytopenia in the same way as localized coagulation, but the likelihood of thrombocytopenia is much greater. DIC may be caused by sepsis, endotoxemia, systemic inflammatory response syndrome, canine IMHA, acute pancreatitis, severe tissue damage (blunt trauma, burns, ischemic necrosis, heatstroke), neoplasms (especially malignant), and some envenomations from various species (e.g., snakes, bees).

(3) Drugs and foreign materials: Some drugs cause or contribute to thrombocytopenia by directly affecting platelets.
  (a) Protamine sulfate, used to reverse the effects of heparin, can induce severe thrombocytopenia in heparinized and nonheparinized dogs, apparently through a direct pro-aggregatory effect.[187]
  (b) BH3 mimetics used to induce apoptosis in cancer cells inhibit the critical prosurvival protein Bcl-xL in platelets, thus leading to apoptosis and thrombocytopenia.[134] This limits the dose at which these drugs can be used.
  (c) Foreign materials used within the vascular system (e.g., tubing and catheters) are tested and chosen to minimize platelet activation but accelerated platelet consumption may occur.

(4) Envenomation (without DIC): The numerous types of venoms from numerous animal species differ in their effects. Venom from some snakes may contain platelet-activating factors that induce thrombocytopenia in the absence of DIC,[188] and thrombocytopenia occurs in a subset of patients with venom-induced consumptive coagulopathy (VICC) (see Chapter 5).[189,190] Phospholipase A2 in many venoms and melittin in bee venom may cause platelet (and RBC) cytotoxicity, apoptosis, and membrane changes that promote accelerated platelet clearance.[191]

(5) Vasculitis or endocarditis: Inflammation of blood vessels or endocardium can alter endothelial cells or lead to exposure of subendothelium, each of which can lead to a prothrombotic state and platelet adhesion, aggregation, and secretion. Vasculitis has infectious, immune-mediated, and chemical causes.
  (a) Infectious causes of vasculitis include Rocky Mountain spotted fever, canine herpesvirus infection, dirofilariasis, angiostrongylosis, infectious canine hepatitis, and equine viral arteritis.
  (b) Thrombocytopenia is commonly associated with bacterial endocarditis, in which case it may have a multifactorial pathogenesis.
  (c) Endothelial cell damage and necrosis lead to thrombocytopenia without DIC in dogs with hemolytic uremic syndrome or cutaneous and renal glomerular vasculopathy, first reported in greyhounds in association with ingestion of Shiga toxin produced by *Escherichia coli*.[192–194] When evaluating greyhounds, it is important to recognize that their platelet concentrations in health are often lower than commonly reported canine RIs.

b. Blood loss, acute and severe

(1) When bleeding accompanies severe thrombocytopenia, hemorrhage is probably secondary to the thrombocytopenia rather than severe thrombocytopenia being caused by the bleeding. However, platelet loss and consumption at sites of hemorrhage may cause or contribute to thrombocytopenia.

(2) In dogs, experimental acute, severe blood loss via phlebotomy with saline replacement of blood volume caused mild to moderate thrombocytopenia (up to 50 % reduction in [platelet]).[195–197]

(3) Thrombocytopenia is sometimes present in bleeding dogs that have anticoagulant rodenticide toxicosis.[198] Platelets are probably consumed at an accelerated rate by use at sites of hemorrhage throughout the body. With extensive hemorrhage, platelet loss from the body also contributes to

thrombocytopenia, and thrombocytopenia may become more severe during routine treatment with fluids, transfusions, and vitamin K_1.

D. Abnormal platelet distribution (sequestration)
   1. Platelets may become reversibly redistributed into the vascular system of certain tissues (e.g., spleen), and therefore the concentration of freely circulating platelets is decreased. This thrombocytopenia occurs without decreased total body platelet mass and therefore does not stimulate thrombopoiesis.
   2. The term *sequestration* is often used for either reversible platelet redistribution or for irreversible trapping of platelets in organs because of destruction by the MPS. However, in this textbook, irreversible removal of platelets from circulation is regarded as decreased platelet survival, not sequestration.
   3. Abnormal platelet distribution may contribute to or rarely cause marked thrombocytopenia.
   4. Disorders
      a. Splenomegaly appears to cause splenic pooling of an exchangeable platelet fraction in some human conditions such as hepatic failure, though platelet survivals may also be decreased.[133,199] Similar effects of splenomegaly may occur in other species.
      b. Severe hypothermia (e.g., a rectal temperature of 20 °C) may cause reversible platelet redistribution to liver and spleen in dogs.[200]
      c. Endotoxemia has been associated experimentally with transient pulmonary platelet pooling in dogs, but platelet consumption also occurs.[201,202]

E. Hemodilution: Massive dilution of blood with platelet-poor fluids (e.g., crystalloids, colloids, plasma, or packed RBCs) may cause mild to moderate decreases in blood [platelet].[203–205]

F. Idiopathic or multifactorial mechanisms
   1. Infections
      a. Infectious thrombocytopenia may be caused by various combinations of suppressed platelet production (e.g., direct infection, immune suppression, or local effects of inflammation within the bone marrow), altered platelet distribution, increased platelet consumption, and immune-mediated or nonimmune platelet destruction, including via increased platelet apoptosis[21] or desialylation by bacterial enzymes.[20]
      b. Thrombocytopenia is commonly associated with endotoxemia[206,207] and infections.[59,72–75,208,209]
      c. Etiologic agents frequently associated with thrombocytopenia include certain bacteria (*Ehrlichia* spp., *Anaplasma* spp., and *Borrelia* spp.),[210] fungi (e.g., *Histoplasma* spp.), viruses (e.g., equine infectious anemia virus), and protozoa (e.g., *Leishmania* spp., *Babesia* spp., *Theileria* spp., and *Trypanosoma* spp.).
      d. Thrombocytopenia occurs somewhat less frequently, but not unexpectedly, with other organisms (e.g., *Leptospira* spp. and feline immunodeficiency virus).[211,212]
      e. *Anaplasma platys* (formerly *Ehrlichia platys*) organisms are rickettsial bacteria that infect platelets and megakaryocytes[213] and cause canine infectious cyclic thrombocytopenia. Infection may be subclinical or have clinical manifestations.[214] Parasitemia may be marked but is often mild, so organisms in blood films (Figure 4.5F) may be difficult to identify definitively. Molecular diagnostic techniques are available[215,216] and have identified *A. platys* in cats, which may not develop clinical disease from the organism.[217] *Anaplasma platys*-like platelet inclusions have been reported (without photographs) in cats, and PCR and sequencing of a blood sample from one such cat yielded a match for *A. platys*.[218]

f. Severe fever with thrombocytopenia syndrome (SFTS) caused by *Dabie bandavirus* (SFTS virus) in the *Phenuiviridae* family is characterized by acute onset of fever, lethargy, gastrointestinal signs, icterus, leukopenia, thrombocytopenia, and often death in people and domestic cats in eastern Asia.[219–221] Fatal disease is also reported in dogs.[222] Evidence for bone marrow suppression and accelerated cell destruction has been reported.[220] Serologic testing supports that many domestic and wild animals are exposed to the virus, likely via ticks, but direct transmission of this zoonotic disease has been reported.

2. Neoplasms[59,223]
   a. Many types of neoplasms are associated with thrombocytopenia, including carcinomas, sarcomas, lymphomas, and leukemias. In one study, approximately 10 % of dogs with neoplasms were thrombocytopenic, though sometimes because of concurrent infections or therapy.[72,76,224]
   b. Thrombocytopenia may result from many mechanisms, alone or combined. In specific cases, causes are usually speculative. Potential mechanisms include the following:
      (1) Decreased production
         (a) Myelophthisis is a presumed contributor to thrombocytopenia in dogs with multiple myeloma, acute leukemia, or chronic lymphocytic leukemia. Other cytopenias are sometimes present.
         (b) Myelodysplastic syndrome may have impaired thrombopoiesis with increased platelet apoptosis.
         (c) Estrogen secretion by a neoplasm may cause myelosuppression.
         (d) Chemotherapy may induce myelosuppression.
      (2) Decreased platelet survival
         (a) Consumptive coagulation occurs in some cancer patients and may contribute to thrombocytopenia seen in dogs that have mast cell neoplasms or hepatic metastases.[224,225] However, most evaluated dogs with neoplasms and decreased platelet survival based on kinetic studies had hyperfibrinogenemia, and fibrinogen half-lives were not decreased.[226,227] This does not support consumptive coagulation.
         (b) Vasculitis or thrombosis within a neoplasm (e.g., hemangiosarcoma, a malignancy of endothelial cells) may accelerate platelet consumption.
         (c) Secondary IMT may also occur and has been incriminated in dogs with certain neoplasms (e.g., lymphoma) by use of indirect platelet and megakaryocyte assays.[148]
         (d) Other contributors may include hemorrhage secondary to a neoplasm, sepsis secondary to immunosuppression, and destruction (phagocytosis) by neoplastic macrophages (e.g., histiocytic sarcoma).
      (3) Abnormal platelet distribution: Splenomegaly or hepatomegaly induced by neoplastic infiltration (e.g., hemangiosarcoma) or secondary organ congestion may be associated with platelet sequestration.
3. Drugs/toxicants: As already noted (Thrombocytopenia, Sections II.B.5, II.C.2.c, and II.C.3.a(3)), the thrombocytopenia associated with drugs may be caused by myelosuppression, accelerated platelet destruction (immune or nonimmune), or multiple mechanisms. In some cases, the pathogenesis of drug-induced thrombocytopenia is not clear, even after extensive investigation.[228]

4. Hypophosphatemia caused by hyperalimentation in starved dogs decreased platelet survival and caused thrombocytopenia.[229]
   a. Thrombocytopenia was associated with decreased platelet ATP concentrations, probably because of an associated decrease in glycolysis. The specific mechanism of accelerated platelet clearance was not determined.
   b. Cats receiving total parenteral nutrition also developed thrombocytopenia, but they were not hypophosphatemic.[230]
5. Anaphylaxis (type I hypersensitivity)
   a. This is an immune-mediated reaction, but the mechanism of thrombocytopenia is incompletely characterized. Nonimmune factors appear to be important in causing thrombocytopenia for at least some hypersensitivity reactions.
   b. Anaphylactic thrombocytopenia may be caused by inflammatory mediators, consumptive coagulation, or immune-complex interactions with platelets. Membrane receptors for immunoglobulin E (IgE) exist on a subpopulation of human platelets and mediate inflammatory platelet reactions in some species.[231]
6. In people with hepatic failure, thrombocytopenia may be caused by decreased Tpo production and splenic sequestration.[133] This may be true in in domestic mammals, but it is not clearly documented.

THROMBOCYTOSIS

I. General concepts
   A. *Thrombocytosis* is a [platelet] greater than a valid upper reference limit, although in some reports about thrombocytosis, the concentration used to define thrombocytosis is somewhat greater than the upper reference limit. Thrombocytosis may result from redistribution or increased production of platelets. Increased production can be associated with hemic neoplasms involving megakaryocytes, or it may occur as a secondary reaction to other conditions. Familial thrombocytoses, including those caused by mutations of the Tpo receptor, occur in people but have not been reported in domestic mammals. However, there may be significant breed differences in platelet concentrations,[66] so comparison to an individual's historical platelet concentrations may be useful in addition to comparison to a routine breed-nonspecific RI.
   B. Appropriate RIs for [platelet] may be shifted upward for individuals at high altitudes.[232]
   C. Serum [K^+] may become increased in vitro (a type of *pseudohyperkalemia*) by the increased amount of platelet K^+ released during clotting.

II. Disorders and conditions (Table 4.3)
   A. Pseudothrombocytosis (spurious thrombocytosis) or false increases in [platelet] can occur when there is an abnormal population of particles with the size and scatter characteristics of platelets. Blood smear examination can confirm thrombocytosis and help exclude causes of pseudothrombocytosis.
      1. Ghost RBCs from intravascular hemolysis (e.g., IMHA, in vitro lysis of RBCs in old samples) may be detected as platelets; instrument platelet scatterplots or differences between impedance and optical platelet concentrations may allow recognition of such an interference.[233,234] Blood smear evaluation can confirm ghost cells unless they form in the analyzer.

Table 4.3. Disorders and conditions that cause thrombocytosis
Reactive thrombocytosis (secondary, nonclonal)
Redistribution
Exercise
Epinephrine
Increased production
Inflammation:* infection, immune mediated disease, surgery, trauma
Nonhemic neoplasms
Iron deficiency*
Vinca alkaloids (vincristine, vinblastine)
Recovery from thrombocytopenia (rebound):* withdrawal of myelosuppression, recovery from IMT
Splenectomy (post)
Starvation
Hemic neoplasms (clonal thrombocytosis)
Primary (essential) thrombocythemia and other chronic myeloproliferative neoplasms
Acute megakaryoblastic leukemia
Myelodysplastic syndrome/myeloproliferative neoplasms

* A relatively common disorder, condition, or mechanism
Note: Pseudothrombocytosis (spurious thrombocytosis) may occur with the presence of many ghost cells (from intravascular or in vitro hemolysis), microcytic RBCs, or certain lipid droplets that are detected as platelets.

2. Microcytic RBCs or potentially marked fragmentation of RBCs can cause pseudothrombocytosis. Cytoplasmic fragments of leukemic cells are unlikely to be numerous enough to cause thrombocytosis, but the [platelet] may be falsely increased by them.
3. Other particles including certain lipids may also cause artifactual thrombocytosis if present in great enough concentration.

B. Reactive thrombocytosis (secondary, nonclonal): Reactive thrombocytosis occurs secondary to one or more other conditions and does not involve neoplasia of the megakaryocyte lineage. The major cause is often unclear because of concurrent contributors, e.g., inflammation and drug therapy associated with a neoplasm, making it unclear if the thrombocytosis relates to the neoplasm, the associated inflammation, the treatment, or some combination of these. Also, in survey studies of thrombocytosis, transient physiologic thrombocytosis could not be excluded in most cases because data were from single CBCs. Persistent thrombocytosis is a nonspecific indicator to assess for an underlying disorder, but the degree of thrombocytosis is not helpful in predicting the cause.[235]
1. Redistribution: Mild and transient physiologic thrombocytosis, even when corrected for hemoconcentration, may occur in some species (e.g., dogs, cats, and people) with strenuous exercise or epinephrine release.[22,236–239] In people, the thrombocytosis appears to result primarily from release of platelets from the spleen, but exercise may induce thrombocytosis in asplenic patients.[240] There also is evidence for platelet redistribution from lungs.[240,241] Phenylephrine given to horses to cause erythrocytosis did not cause concurrent thrombocytosis.[24]

2. Increased production
   a. Inflammation: Inflammatory cytokines, including IL-6, stimulate Tpo production by hepatocytes and thus increase thrombopoiesis in a wide variety of infectious and noninfectious inflammatory conditions, including surgical and nonsurgical trauma in people.[2,242,243] This may drive plasma [Tpo] higher than expected for the magnitude of the [platelet]. Infectious and noninfectious (e.g., immune-mediated) inflammatory disease is one of the most common categories of disorders associated with thrombocytosis in domestic mammals.[235,244–247]
   b. Malignant neoplasms: Thrombocytosis may result from accompanying inflammation or from production of thrombopoietic cytokines (e.g., IL-6, Tpo) by neoplastic cells.[248–250] Various carcinomas, lymphomas, and mast cell neoplasms have been most commonly associated with thrombocytosis in dogs, but thrombocytosis occurs in dogs with many types of neoplasm.[235,246] There is less information for cats.[247]
   c. Iron deficiency: Thrombocytosis is a common but inconsistent finding in canine and human patients with iron deficiency. Blood concentrations of measured thrombopoietic cytokines (e.g., IL-6, granulocyte-macrophage colony-stimulating factor, or Tpo) have not been increased in human patients with iron deficiency and thrombocytosis, and evidence does not support a cross-lineage effect of Epo.[251,252] Instead, murine studies support that low marrow iron concentration alters the metabolism of erythroid/megakaryocyte progenitors (ErMegPr in Figure 2.1) and promotes their commitment to the megakaryocyte lineage at the expense of the erythroid lineage.[253] The increased throughput of megakaryocytes is associated with increased ploidy and increased proplatelet production.
   d. *Vinca* alkaloids: Vincristine and vinblastine stimulate thrombopoiesis that can lead to thrombocytosis without increased MPV.[254,255] In patients with IMT, these drugs may also promote increased platelet concentrations by decreasing platelet destruction via inhibition of the MPS.
   e. Recovery from thrombocytopenia (rebound thrombocytosis): Thrombocytopenia may stimulate enough thrombopoiesis that production exceeds consumption or destruction, and blood concentrations transiently overshoot the upper reference limit during recovery. This can occur after withdrawal of myelosuppression, with recovery from IMT, or after blood loss.[195,256–258]
   f. Postsplenectomy: Splenectomy causes thrombocytosis, sometimes marked, associated with increased thrombopoiesis and increased blood concentrations of Tpo.[259–261] Thrombocytosis is transient but may persist for weeks. Other mechanisms that may contribute to the thrombocytosis include decreased platelet destruction (longer life span), decreased sequestration,[262] or rebound thrombopoiesis if a consumptive or destructive thrombocytopenia was present at the time of splenectomy.[263]
   g. Blood loss, especially chronic, has been associated with thrombocytosis in several species, but iron deficiency, inflammation, rebound thrombocytosis, or neoplasms may be the specific causes or contributors to thrombocytosis in many of these cases. Thrombocytosis associated with blood loss may require the presence of a spleen; it occurred with repeated phlebotomy (chronic blood loss) in nonsplenectomized rabbits that were supplemented with iron, but did not occur in iron-supplemented, splenectomized rabbits.[264]
   h. Hypercortisolemia: Hyperadrenocorticism and exogenous glucocorticoids have been associated with thrombocytosis in dogs,[244,246] but a cause-and-effect

relationship is not clear. Thrombocytosis may relate to underlying or concurrent conditions such as inflammatory disease or neoplasia. Prednisone administration to healthy dogs resulted in no increases[254] or questionable increases[265] in platelet concentrations. In an unpublished study of the effects of an immunosuppressive dose (2 mg/kg) of prednisone on canine platelet function (MAS), prednisone given daily to six dogs over 21 d did not have a demonstrable or consistent effect on [platelet] and did not increase it compared to two control dogs.

i. Starvation: Thrombocytosis is common and likely multifactorial in starved dogs.[266]
j. Other disorders have been present in dogs and cats with thrombocytosis, but the relationships and potential causes are less clear or consistent. These include renal disease, cardiac disease, and diabetes mellitus in dogs,[235,246,267] and hyperthyroidism in cats.[247]

C. Hemic neoplasms (clonal thrombocytosis)
1. Primary (essential) thrombocythemia (see also Chapter 6): This is a rare chronic myeloproliferative neoplasm that has been reported in a few dogs and cats.[268] Platelet concentrations have been persistently and markedly increased (1,000,000–5,000,000/μL) (Figure 4.3C). Pleomorphic large and/or hypogranular platelets may be present and similar to those seen with some megakaryocytic leukemias (Figure 4.6). PDW may be increased. Increased numbers of mature megakaryocytes are in the bone marrow (megakaryocytic neoplasia, not hyperplasia), and megakaryocytes may have atypical features.
   a. The human condition involves a clonal proliferation of pluripotent stem cells.[269] Diagnosis in domestic mammals is currently by exclusion of other causes of persistent thrombocytosis, although one could assess for mutations in the commonly affected genes in people with essential thrombocythemia: *JAK2*, *CALR*, and *MPL*.
   b. Data are lacking regarding the prevalence of thrombotic or hemorrhagic tendencies in domestic mammals with clonal thrombocytosis.
2. Other chronic myeloproliferative neoplasms: polycythemia vera, primary erythrocytosis, and chronic myeloid leukemia
3. Acute megakaryoblastic leukemia (M7 subtype of acute myeloid leukemia) has been reported rarely in dogs and cats (Figure 4.6). Thrombocytosis or thrombocytopenia may be present. In this myeloid leukemia, in contrast to essential thrombocythemia, ≥ 20 % of nucleated cells in the marrow are megakaryoblasts with ≥ 50 % of nucleated cells having a megakaryocytic phenotype (World Health Organization criteria); marrow fibrosis may be present and neoplastic megakaryoblasts may be present in blood and other organs.[127,128,270–272]
4. Other myeloid neoplasms such as myelodysplastic syndrome/myeloproliferative neoplasms are sometimes associated with thrombocytosis.[273]

INTERPRETATION OF MPV, PDW, PCT, AND MPC

I. Interpretation of platelet indices is limited by platelet clumping, inaccuracies and inconsistencies of routine measurements, and limited knowledge of pathophysiologic factors influencing platelet size,[46,51,274] especially in domestic species. Anticoagulant, storage time, storage temperature, and analyzer all affect the results. Values should be interpreted relative to RIs generated from samples handled in the same manner as the patient samples. Many reports about significant increases or decreases in values have been based on statistically significant differences among

groups rather than on increases or decreases compared to reference limits. Commonly, with impedance analysis and thrombocytopenia, abnormal platelet volume distributions result in the analyzer not reporting MPV, PDW, and Pct, so these parameters may not be available for interpretation when they might be of most interest.[44]

II. MPV: Among individuals, populations, and species, MPV is generally inversely related to [platelet],[275] and MPV values tend to be greater with thrombocytopenia and lower with thrombocytosis.[276] A similar relationship was found in healthy cats.[277] Because MPV is an average, populations of large or small platelets may be present and detectable by microscopy when the MPV is WRI. Also, large platelets may be seen microscopically but excluded from analysis by the instrument because they are larger than the upper threshold set for platelets. Potential causes of such a population are similar to those for an increased MPV.
   A. Increased MPV (without platelet clumping)
      1. In vitro platelet swelling and activation changes may increase the MPV over time (see Quantitative methods, Section C.5), although the decrease in light scatter because of H_2O uptake and diluted cytoplasmic contents can yield a falsely low MPV with single-angle optical analyzers.[56] Some of the platelet changes that occur in EDTA tubes over time appear to be the result of platelet activation, and use of platelet inhibitors diminishes the increase in MPV.[49] Addition of thrombin to EDTA-anticoagulated blood, together with an inhibitor of platelet aggregation to prevent clumping of activated platelets, increased MPV (and decreased MPC).[31,50] Therefore, in vitro or in vivo platelet activation may increase MPV, but in states of platelet activation, an increase in MPV also may be caused by production of large platelets, and in vitro activation may generate small platelet clumps that falsely increase MPV.
      2. Accelerated thrombopoiesis caused by an increased stimulus for platelet production,[14,46] typically associated with increased megakaryopoiesis (megakaryocytic hyperplasia)
         a. Stimulated platelet production can occur with increased hepatic removal of desialylated platelets, decreased platelet/megakaryocyte mass, or increased cytokine-driven Tpo production. Platelet binding of von Willebrand factor or certain antiplatelet antibodies may induce platelet desialylation that promotes clearance via Ashwell–Morell receptors, thus increasing Tpo production. Certain bacteria may promote similar clearance by releasing sialidases that deglycosylate platelet surface proteins.[20] Activated platelets can release intact Tpo in vivo, thus increasing blood concentrations of Tpo and promoting thrombopoiesis during platelet-consumptive states.[278]
         b. In people, MPV tends to be greater in patients with IMT, which is caused by shortened platelet survival and impaired thrombopoiesis, than in patients with thrombocytopenia caused primarily by bone marrow disease (e.g., aplastic anemia), though it may be increased with either.[51] In dogs with IMT, MPV has been increased, decreased, or WRI.[279,280]
         c. Although there are reports to suggest postproduction size modification of platelets,[258] platelet size is generally thought to be established primarily during production and not dramatically altered in the circulation.[46,281] Therefore, increased MPV may reflect accelerated thrombopoiesis occurring any time within 5–10 d (platelet life span in health) before testing. Increased MPV may precede increases in [platelet] in patients recovering from thrombocytopenia;[46] the same is true for RPlt%.[14]

d. In general, larger platelets are associated with more organelles, more surface area, and greater functional capacity.[51] This is reflected by an increased MPM (dry-weight mass of the average platelet in the sample) if the platelets have not been activated. It also likely explains why people with thrombocytopenia and IMT (with increased MPV) have shorter bleeding time than people with similar degrees of thrombocytopenia but lower MPVs.

3. Increased production of abnormal platelets in essential thrombocythemia and other clonal disorders may lead to an increased or unexpectedly high MPV for the degree of thrombocytosis.[46,282]
4. Abnormally large platelets are produced in certain congenital platelet disorders, so MPV may be increased. Exclusion of large platelets may underestimate the MPV.
   a. Large platelets were reported in otterhounds with Glanzmann thrombasthenia.[283]
   b. They are present in many Cavalier King Charles spaniels and other dogs with a defect in β1 tubulin synthesis (Figure 4.5D and E).[64,79–81,284] The resulting deficiency of platelet microtubules impairs proplatelet formation and thrombopoiesis.
   c. May–Hegglin anomaly caused by a mutation in the gene for myosin 9 (nonmuscle myosin heavy chain IIa) was diagnosed in an 8-yr-old pug with asymptomatic macrothrombocytopenia and neutrophil inclusions typical of the anomaly in people (see Chapter 2).[83]
   d. Increased MPV, mild to moderate thrombocytopenia, and a bleeding tendency occur in dogs with Bernard–Soulier syndrome, a genetic disorder causing reduced expression of the platelet membrane receptor (glycoprotein Ib-IX) for von Willebrand factor.[62,285] Although reported as "markedly large," the platelets described in affected dogs generally had diameters less than those of RBCs (on blood films) and were not large enough to interfere with detection or to necessarily be reported as macrothrombocytes. Giant platelets do occur in human Bernard–Soulier syndrome. Affected dogs have been Cocker spaniels of European origin with a large deletion in the *GP9* gene for glycoprotein IX.
   e. Macrothrombocytopenia of undetermined cause was reported in few Akitas with elongate platelets or proplatelets.[60]
5. MPV may increase with physiologic thrombocytosis, possibly because of mobilization of the splenic platelet pool, which is thought to be overrepresented by large platelets in some species.[46]
6. Acute infection with the Kawakami–Theilen strain of feline leukemia virus induced production of macrothrombocytes.[286] This was associated with decrements in [platelet] but no significant change in Pct.

B. Causes of decreased MPV
1. MPV tends to be increased in human patients with IMT and has been similarly increased in dogs with primary IMT,[279] but, in other reports, MPV has been WRI or decreased in dogs with IMT.[287,288] Differences may relate to methodologic issues or disease states. Increased numbers of platelet microparticles are detected by flow cytometry in the blood of dogs with IMT.
2. With severe thrombocytopenia ($< 5 \times 10^3/\mu L$), MPV may be affected by nonplatelet debris in the sample or instrument (e.g., carryover from a previous sample) if special precautions are not taken. This debris is insignificant except at very low platelet concentrations when it can affect values from light scatter or impedance methods.

3. Bone marrow failure and chemotherapeutic myelosuppression have been associated with decreased MPV in people, but MPV is not always decreased.[282,289] MPV was WRI for seven of nine thrombocytopenic dogs diagnosed with primary bone marrow disease and was increased in two of nine, neither of which had megakaryocytic hypoplasia.[287]
4. In people with severe thrombocytopenia, the risk of hemorrhage is greater when the MPV is decreased.[51]

III. PDW: An increased PDW indicates an increased population of large platelets, an increased population of small platelets, or both. Factors affecting the reliability of platelet volume measurements (e.g., platelet clumping, sample age) also affect PDW. In people, thrombocytosis with a PDW that is WRI is suggestive of a reactive thrombocytosis rather than a myeloproliferative neoplasm.[51]

IV. Pct: Compared to the [platelet], Pct may better represent the total platelet functional potential and the thrombopoietic stimulus.[275]
   A. This was true in Cavalier King Charles spaniels, although macrothrombocytes that are too large to be recognized as platelets may falsely lower the Pct in these dogs, while still providing a better assessment of primary hemostatic potential than [platelet].[37] In other words, [platelet] appeared decreased in many more Cavalier King Charles spaniels than did the Pct.
   B. In dogs with presumptive primary IMT, Pct returned more quickly than [platelet] to equivalent thresholds of recovery because MPVs tended to be increased.[279] The Pct recovery is thought to be a better measure of hemostatic potential because of the greater reactivity of large platelets.
   C. False decreases in Pct are expected with significant platelet clumping given the greater decrease in [platelet] compared to increase in MPV, but data were not found.

V. MPC: In theory, if platelets are spheroid, MPC can be used to detect pathologic states associated with platelet activation.
   A. With in vivo platelet activation, granule secretion, and uptake of H_2O, refractive index and therefore MPC decrease.[31,45,55] However, MPC also decreases with in vitro swelling or activation induced by collection and handling, and platelets in routine EDTA samples undergo activation changes as they age: decreased α granules, increased MPV, decreased MPC, dilated open canalicular system, and clumping.[49] Decreases in MPC were reduced by storage at 4 °C compared to room temperature. Use of platelet signaling pathway inhibitors helped stabilize platelets and MPC,[49] as did use of CTAD anticoagulation.[31]
   B. Platelets tend to become spheroid in EDTA, but if they remain more discoid, light scatter does not adhere to Mie theory, and MPC is difficult to interpret. A more spheroid population is supported by a more uniform platelet component and therefore a lower PCDW (reportedly < 5.5 g/dL in dogs),[50,55] although PCDW could be increased with a spheroid population. Platelet activation associated with routine collection and use of EDTA tubes limits the potential value of MPC; volume and refractive index measures are approximations.
   C. In one study, some dogs with nonseptic or septic inflammatory disease had decreased MPC, and MPC correlated, though not strongly, with expression of the activation marker P-selectin.[290] The dogs with inflammation also tended to have lower platelet concentrations and increased MPV, PDW, and PCDW. However, in another retrospective study, MPC was not decreased in dogs with various inflammatory diseases compared to dogs

needing orthopedic surgery for traumatic or degenerative disorders, with the exception of dogs with IMT.[280] And in a retrospective study of dogs with IMHA, nonIMHA illness, or good health (95 dogs in each group), MPC was lower in IMHA dogs but was decreased in only a minority; most values were WRI and there was a rare increased value.[291] About two-thirds of the IMHA dogs had spherocytosis, and there was no indication of whether platelet scatterplots had been assessed for ghost cells (formed in vivo or in the analyzer) or if ghost cells could have interfered with platelet indices using that particular software.

RETICULATED PLATELETS AND IMMATURE PLATELET FRACTION

I. Reticulated platelets are young platelets containing increased RNA, which can be seen microscopically as reticular blue staining with new methylene blue vital staining.[195] They are analogous to reticulocytes in terms of age, but they are functionally mature.
   A. Assessment has been primarily by flow cytometry and for research purposes, but a similar assessment is done with certain automated hematology analyzers, some of which report "immature platelet fraction" (IPF) rather than "reticulated platelet" results. Values for immature platelet fraction are enough different from flow cytometric reticulated platelet results that these terms are often distinguished from each other (not used interchangeably).
   B. RPlt% or IPF%
      1. Percentage of platelets that have increased RNA
      2. Unit: %
   C. [RPlt] or [IPF]
      1. Concentration of reticulated or immature platelets in blood calculated as the product of [platelet] and platelet reticulocyte percentage or immature platelet fraction (in decimal form)
      2. Units: $(\# \times 10^3/\mu L) \times 10^6\ \mu L/L = \# \times 10^9/L$ (SI unit)

II. Methods
   A. Flow cytometry: Platelets are typically incubated with thiazole orange, a fluorescent dye that binds to platelet RNA but can also bind to granule nucleotides and mitochondrial DNA. Concurrent use of a labeled antiplatelet (e.g., anti-CD61) antibody allows selective evaluation of platelets, which can overlap in light scatter with RBCs, particularly when there are macrothrombocytes and/or microcytic RBCs. The percentage of platelets with increased fluorescence is determined, and a [RPlt] can be calculated.
   B. Automated hematology analyzers: Immature platelet results generated by automated hematology analyzers have not been interchangeable with each other or with flow cytometric results because of differences in methods, including dyes, which have different binding specificities.[14,292,293] Of analyzers with the capability to assess immature platelets, the Sysmex XT-2000iV and the ADVIA 2120 (software version v6.11.7) have been evaluated for canine samples.[293,294] Further studies are needed to determine the diagnostic utility of these methods.
      1. Sysmex XR-2000iV: Polymethine, a fluorescent dye, binds to platelet nucleic acids and is detected in the instrument's optical channel, which concurrently assesses cell volume by light scatter. Manual gating allows exclusion of most RBCs, which may nonspecifically bind polymethine, and of reticulocytes, which contain RNA. WBC fragments, which may be relatively prevalent in some patients with leukemia, may be detected as reticulated platelets.

2. ADVIA 2120 (software version v6.11.7): Oxazine 750 binds to nucleic acids and is detected spectrophotometrically (absorption) in the reticulocyte channel while particle volume and refractive index are concurrently assessed by light scatter. Manual adjustment of the threshold for platelet immaturity after visual assessment of scattergrams allowed inclusion of otherwise excluded reticulated platelets and exclusion of microcytic RBCs.

C. An increase in fluorescence or absorption may relate to an increase in platelet size (more mitochondrial nucleic acid and dense granule nucleotides) or to an increase in RNA concentration within young platelets, depending on how the threshold for increased signal is defined and the method used (e.g., dye, dye concentration, incubation time, or fresh versus fixed samples).[13,292,295–297] RPlt% also increases with sample fixation and with sample storage at either 4 °C or 21 °C, so prompt testing and parallel control samples are recommended;[13,298] increases are common by 18 h, but may occur sooner with certain samples. Platelet clumping, including very small clumps, appears to falsely increase RPlt%.[299]

III. Interpretation

A. Reticulated platelet results directly reflect thrombopoiesis and indirectly reflect megakaryopoiesis. Thrombopoiesis can be impaired when megakaryopoiesis is increased, and an increase in reticulated platelet measures supporting increased thrombopoiesis can be present with megakaryocytic hypoplasia. Also, RPlt% may be increased when MPV and the platelet large cell fraction are not.[298]

B. The clinical utility of young platelet assessment in domestic mammals is not clearly established. Results and reference values vary considerably with the instrument and method, including gating strategies, and assay imprecision can be quite high.[293,298] Variations in methods and uncertainty about the impact of platelet size (versus just age) on young platelet detection complicate interpretation of reported findings.

C. Values have usually been reported and interpreted as percentages rather than as concentrations. However, this can be misleading, and percentage data should always be interpreted with consideration of the [platelet] and whether there is a thrombocytopenia or thrombocytosis.

1. With thrombocytopenia:
   a. Analogous to interpretation of immature RBCs (reticulocytes), increases in the RPlt% or IPF% may be relative and not truly indicative of increased platelet production; the release of young platelets may be unchanged, but because of fewer platelets in circulation, their percentage is increased without an increased concentration (at the extreme, 100 % of 0/μL is 0/μL).[14,169]
   b. However, with severe thrombocytopenia, the percentage of young platelets may be increased during an appropriate thrombopoietic response when their concentration is not, because there may simply be too few platelets for any percentage of them to be increased (again, a high percentage of a very low concentration is a low concentration).[169,300] In addition, concentrations of platelets, including young platelets, may be further suppressed because platelet turnover is increased, whatever the cause of severe thrombocytopenia; the relatively few platelets, younger and older, are consumed at an accelerated rate to maintain normal vascular integrity.[19,301]
2. With thrombocytosis: An increased [RPlt] or [IPF] can be present without an increased RPlt% or IPF% because a "normal" percentage of a greater [platelet] is a greater

concentration. The greater [RPlt] or [IPF] indicates greater thrombopoiesis, which would be missed by evaluating only the RPlt% or IPF%.

D. Causes of increased [RPlt], [IPF], RPlt%, or IPF%
   1. In general, these findings suggest stimulated thrombopoiesis and accelerated or increased production of platelets from megakaryocytes, a process which is often associated with megakaryocytic hyperplasia, but which also occurs with megakaryocytic hypoplasia. The more megakaryocytes there are producing platelets, the greater the [RPlt] or [IPF] can become, but the RPlt% and IPF% can be substantially increased with moderate or even decreased megakaryocyte mass.
   2. Thrombocytopenia:
      a. Increased RPlt% has been present, when evaluated, in most dogs and horses with thrombocytopenia caused by decreased platelet survival.[169,298] In contrast, it was not increased in four dogs with carboplatin-induced myelosuppression.
      b. However, [RPlt], [IPF], RPlt%, or IPF% may be increased in production-failure thrombocytopenias such as aplastic anemia and myelophthisis, so increases do not confirm adequate thrombopoiesis or exclude various causes of bone marrow failure.[292,302] While some studies suggest that increases are less with such disorders as compared to thrombocytopenia caused by decreased platelet survival,[303] the overlap in IPF% increases among human patients with IMT or various bone marrow failure disorders was large in other studies, providing limited or no discriminatory power to distinguish one cause of thrombocytopenia from another.[304]
      c. In a study of thrombocytopenic dogs with decreased to increased megakaryocyte densities (number) in bone marrow aspirates, RPlt% increased similarly with decreased or increased megakaryocyte density.[305] The [RPlt] also increased with both groups but was significantly more increased in dogs without megakaryocyte hypoplasia. Therefore, the [RPlt] better reflected megakaryocyte density (number). These findings support that RPlt% and [RPlt] may be increased with megakaryocytic hypoplasia, reflecting stimulated thrombopoiesis despite what might be an insufficient number of megakaryocytes to maintain the [platelet] WRI (decreased platelet production is contributing to the thrombocytopenia despite increased production by the megakaryocytes that are present).
   3. Thrombocytosis: Although it has been suggested that reticulated platelet assessment can help differentiate reactive thrombocytosis from primary thrombocytosis, increases in flow cytometric RPlt% were similar in people with primary thrombocytosis, reactive thrombocytosis, thrombocytopenia from decreased platelet survival, or recovery from chemotherapeutic myelosuppression.[306] Because Tpo is a major stimulus for thrombopoiesis, this aligns with the finding of similar serum Tpo concentrations (WRI or increased) in people with primary thrombocytosis and those with reactive thrombocytosis.[307]
   4. Recovery from bone marrow suppression: In people, increases in RPlt% precede recovery of [platelet] in patients recovering from chemotherapy-induced myelosuppression.[14,302,306]
   5. Macrothrombocytopenia: Depending on specific methods, macrothrombocytes may be detected as young (reticulated) platelets throughout their life span, leading to false increases in young-platelet identification and overestimation of thrombopoiesis.[299]
   6. Erythropoietin administration to dogs was associated with increased RPlt% and platelet hyperreactivity.[308]

E. Decreased [RPlt], [IPF], RPlt%, or IPF% supports decreased thrombopoiesis, but some lower reference limits do not allow detection of decreases.

ANTIBODY TESTS FOR IMMUNE-MEDIATED THROMBOCYTOPENIA (IMT)

I. Clinical indications for assessing antiplatelet antibodies include assessing for posttransfusion purpura (rare), neonatal alloimmune thrombocytopenia (rare), platelet refractoriness from transfusion-induced alloantibodies (rarely needed), and potentially primary or secondary IMT (including evaluation for relapses and for drug-induced IMT). Before testing for IMT, thrombocytopenia should be confirmed with blood smear review of samples lacking significant platelet clumping. However, positive results with veterinary assays do not differentiate autoantibodies from alloantibodies or immune complexes (hence the term platelet-associated antibodies rather than antiplatelet antibodies), and they do not differentiate primary IMT from secondary IMT. Furthermore, the diagnosis of primary IMT is by exclusion; a negative test for antiplatelet antibodies, even by a well-validated test, does not exclude IMT, though a positive result from a well validated assay supports the conclusion of an immunologic component to the thrombocytopenia. Despite human assays being much better developed and more thoroughly evaluated than veterinary assays, antiplatelet antibody testing is of unproven benefit and therefore has not been recommended as a routine test for the diagnosis of primary IMT in people.[150]

II. A variety of platelet antibody assays have been developed for dogs, while use of assays for cats and horses has been more limited. Platelets are relatively difficult to work with, so platelet assay development and implementation requires particular experience with platelets of relevant species to avoid mishandling samples, activating platelets, and detecting nonpathologic platelet-associated IgG (e.g., IgG released from α granules or cold-reacting serum antibodies that bind to platelets in vitro but were not bound in vivo). Most current veterinary assays are flow cytometric, but there are significant differences in methods, from sample processing through the criteria used to establish positivity and the quality and suitability of RIs. This limits our understanding of platelet-associated antibodies and makes interpretation of results and published data more difficult.

III. Platelet antibody assays may be direct or indirect.
   A. Direct assays test for antibodies on the surface of a patient's platelets (PSAIg). With flow cytometric assays, PSAIg is detected by fluorescence-tagged, species-specific, anti-immunoglobulin antibodies.[144,149,159,161,164,169,288,309–313]
      1. Sample: whole blood anticoagulated with EDTA or citrate
      2. Sample processing: Platelets are usually separated by differential centrifugation and then washed. Centrifugation methods, storage temperature, washing temperatures, and washing techniques all vary, and some may cause substantial platelet activation and alterations that affect results. Platelet activation during washing can be inhibited by addition of prostaglandin E1. Accepted storage times prior to testing also vary. The effects of permutations of these variations on assay results require further investigation, but some effects are known.
         a. With a well-characterized immunoradiometric assay, exposure of citrated blood to room temperature or refrigeration for a few hours led to variable but sometimes marked increases in PSAIg in canine samples.[146] So, samples were kept warm and maintained at 37 °C until centrifugation and washing with warmed buffer. In

general, platelets are more disturbed by refrigeration than by short periods of room temperature storage or exposure to 37 °C.

b. Storage-related increases in PSAIg also occur with EDTA anticoagulation,[314] but the relative contribution of anticoagulant, storage temperature, and other factors to these increases is not known. Loss of CD61 detection over 8–36 h was noted in a flow cytometric assay of samples stored at 4 °C, particularly in the group of dogs with the most severe thrombocytopenia and diagnosed with primary IMT.[312] Scatterplots of positive samples lacked distinct platelet clouds, suggesting that platelets had been altered during processing.

c. Marked scattering of the platelet "cloud" in light scatterplots used in flow cytometric tests signals a problem that may involve platelet activation or damage and result in inaccurate results.

3. Analysis: Typically, standard volumes and concentrations of washed platelets are incubated with labeled antibodies to a platelet marker (e.g., CD61) and various labeled antibodies reactive to either IgG or IgM that is being detected. After gating on platelets (particles with the appropriate light scatter characteristics that are labeled with the antiplatelet antibody), fluorescence caused by bound anti-immunoglobulin antibodies is assessed and compared to various negative control or reference samples. The method for determining positivity varies, but a cutoff is determined that attempts to separate negative from positive. Differentiating a true weak positive result from a truly negative sample can be impossible.

4. Reference values: These should be established with identical sample handling as used for patient samples. There can be procedural variations between reference and later test samples that appear minor but result in significant effects on results. For one PSAIg assay, RIs had to be redone when it was realized that a difference of less than an hour in the time from collection to harvesting platelets significantly altered the results.[146] Reference samples had been processed more quickly because the samples came from a building adjacent to the laboratory, while patient samples were cooling in transit from a building a block away, resulting in increased PSAIg.

5. When positive results are generated from properly processed samples by using reliable assays, they indicate that increased antibody is associated with the platelet surface and may be contributing to antibody-mediated platelet destruction. The antibodies could be autoantibodies, alloantibodies, immune complexes bound via immune adherence in some species (likely dogs) or potentially Fc receptors in some species (likely not dogs), or immunoglobulin bound in some nonspecific manner. Positive results in nonthrombocytopenic patients should be interpreted with caution.

6. Negative results suggest the need to search for a nonimmune pathogenesis for the thrombocytopenia, but negative results may occur if (i) complement or an isotype not tested for is mediating the thrombocytopenia (e.g., IgA with an assay for IgG), (ii) the amount of antibody is below an assay's detection limit, or (iii) cell-mediated immunity is the principal problem.

B. Indirect assays assess for antibodies in a patient's plasma or serum that can bind to platelets (or, in human medicine, isolated platelet epitopes) from healthy animals. Positivity shows that a sample contains antibodies that can bind to platelets under test conditions, and therefore provides indirect evidence that plasma antibodies may be binding to a patient's platelets in vivo.

1. However, acquired or naturally occurring alloantibodies to platelet antigens, immunoglobulin aggregates formed in vitro (e.g., with sample freezing),[315] antibodies

that bind to EDTA-induced neoepitopes on test platelets, and possibly cold-reactive immunoglobulins may yield positive results that do not reflect an immune-mediated attack on patient platelets.

2. Conversely, false negative results may occur when antigenic sites are altered by EDTA anticoagulation and dissociation of $\alpha_2\beta_3$.[316]
3. With well-characterized human assays, indirect testing has been shown to have less diagnostic sensitivity and specificity for primary IMT than direct assays. However, it is useful for detection of alloantibodies and drug-induced antiplatelet antibodies, and to provide screening evidence of IMT. Positivity in nonthrombocytopenic dogs is difficult to interpret but suggests that the detected antibody is not mediating platelet destruction in vivo.

C. As with any assay, use of negative and positive controls is standard good laboratory procedure to assess for analytical problems. While negative control samples are routinely employed, positive control samples are more difficult to generate and have often been omitted. However, alloantibodies,[310] a characterized canine autoantibody,[146] immunoglobulin (mixed IgG and IgM) bound to platelets by immune adherence,[317] and silica microspheres coated with IgG or IgM[318] have been used. The allo- and autoantibodies can be used as positive control antibodies in indirect assays, and they can be incubated with platelets to generate antibody-coated positive control platelets for direct assays. However, availability and reproducibility are limitations.

IV. Direct megakaryocyte immunofluorescence assays have been used to detect the presence of megakaryocyte-associated immunoglobulins on a patient's megakaryocytes, usually on smears of a bone marrow aspirate.[176,319] Testing requires good bone marrow aspirates with ample megakaryocytes, so samples are not always adequate. If antibodies directed against platelets also bind shared epitopes on megakaryocytes, as is expected, results may be positive.[320] False-positive results may occur if megakaryocytes are damaged and cytoplasmic immunoglobulin rather than surface immunoglobulin is detected; the amount of IgG in α-granules is large compared to PSAIg in IMT. The cumulative reported diagnostic sensitivity for clinical diagnoses of canine IMT is about 50 %, which is too low to recommend.[148]

References

A complete set of references is available online. Please use the below link to access the References files.

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HEMOSTASIS

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Table 5.1. Abbreviations and symbols in this chapter

[x]	x concentration (x = analyte)
ACT	Activated coagulation (clotting) time
ADP	Adenosine diphosphate
APC	Activated protein C
AT	Antithrombin (antithrombin III)
BMBT	Buccal mucosal bleeding time
Ca^{2+}	Calcium
DDAVP	1-deamino-8-D-arginine vasopressin
DIC	Disseminated intravascular coagulation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
fCa^{2+}	Free ionized calcium
Fbg	Fibrinogen
FI–FXIII	Inactive coagulation factors 1–13; terminal "a" (e.g., FIIa) denotes activation
FDPs	Fibrin or fibrinogen degradation products; fibrin fragments + fibrinogen fragments
GPIb	Glycoprotein Ib (CD42b)
Hct	Hematocrit
HK	High molecular weight kininogen
INR	International normalized ratio
ISI	International Sensitivity Index
LMWH	Low molecular weight heparin
MPS	Mononuclear phagocyte system
M_r	Relative molecular mass
PAI	Plasminogen activator inhibitor
PIVKA	Proteins induced by vitamin K antagonism or absence
PK	Prokallikrein or prekallikrein (synonyms); not pyruvate kinase (Chapter 3)
PT	Prothrombin time
PTT	Activated partial thromboplastin time
RVVT	Russell's viper venom time (dRVVT = dilute RVVT)
SI	Système international d'unités (International System of Units)
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin–antithrombin complexes

Table 5.1. Abbreviations and symbols in this chapter (Continued)	
TBT	Template bleeding time
TF	Tissue factor, tissue thromboplastin factor
TFPI	Tissue factor pathway inhibitor
t-PA	Plasminogen activator, tissue type
TT	Thrombin time
TT_{Clauss}	Modified TT for [Fbg] by using the method of von Clauss
u-PA	Plasminogen activator, urokinase type
VET	Viscoelastic testing
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:Ag	[VWF] detected antigenically; also describes the assays used for this measurement
VWF:CB	[VWF] detected antigenically in collagen binding assays; also describes such assays
WRI	Within reference interval

HEMOSTASIS

I. *Hemostasis* is the arrest of bleeding or the interruption of blood flow through a vessel. The term is also used more generally to refer to the intricate and balanced physiologic processes that maintain blood in a freely flowing state but allow the rapid formation of localized solid plugs to seal injured vessels. Normal hemostasis depends on the complex continuous interactions of its major components: platelets, coagulation factors, anticoagulants, pro- and antifibrinolytic factors, and blood vessels.
   A. Platelets constantly survey and repair vascular microdefects, promote localized coagulation by forming primary hemostatic (platelet) plugs, and release factors that influence other platelets, coagulation, fibrinolysis, and blood vessels.
   B. Plasma procoagulant and anticoagulant factors constantly circulate in nonactivated forms that can be rapidly activated to provide localized secondary hemostatic plugs containing fibrin to stabilize platelet plugs. Some coagulation factors promote fibrinolysis; some inhibit it.
   C. Fibrinolysis helps control the extent of coagulation through a balance of pro- and antifibrinolytic factors; it also has pro- and antiplatelet effects.
   D. Blood vessels influence the hemostatic process through endothelial cell properties, effects of exposed subendothelium, and alterations in blood flow. These impact platelet function, coagulation, anticoagulation, and fibrinolysis.

II. The hemostatic response to vascular injury is generally divided into three major processes, but they actually occur concurrently with checks and balances to control the process (Figure 5.1).
   A. *Primary hemostasis* is the development of a relatively unstable primary hemostatic plug (platelet plug) at the site of vascular injury (Figure 5.1A).
   B. *Secondary hemostasis* is coagulation and formation of a stable secondary hemostatic plug within and around the primary hemostatic plug (Figure 5.1B). This plug is a *thrombus*, a mass of clotted blood, platelets, and trapped RBCs and WBCs adherent to the vessel wall. A thrombus may obstruct blood flow. A piece that breaks from a thrombus and flows elsewhere is an *embolus*, and that process is *thromboembolism*. Although thrombi are

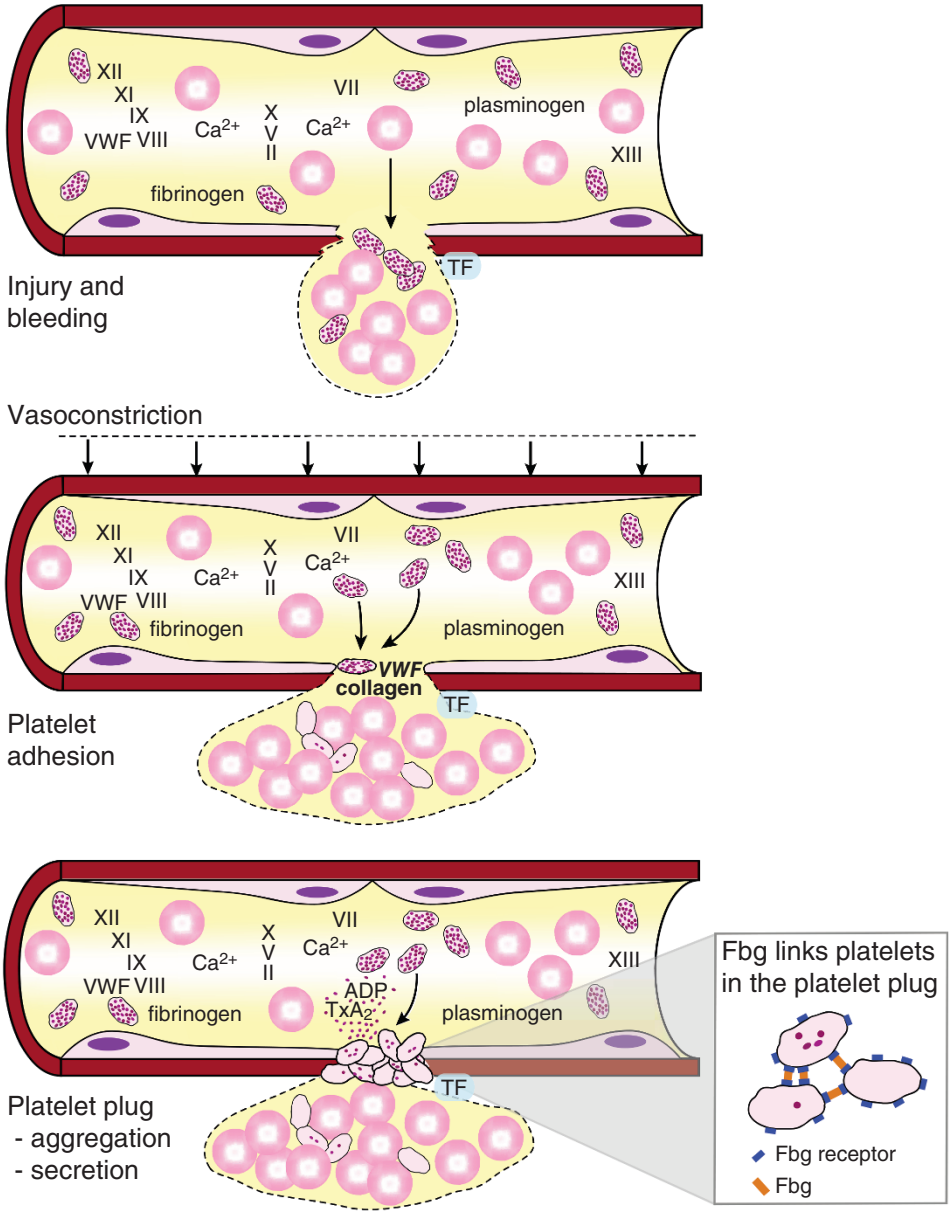


Figure 5.1A. The hemostatic process: vasoconstriction and primary hemostasis after vessel damage.

1. Vasoconstriction: Rapid, transient vasoconstriction (*arrows indicate vessel narrowing*) helps limit blood loss. Vasoconstriction has both neurogenic and vasoactive components.
2. Platelet adhesion: Circulating or subendothelial VWF secreted by endothelial cells binds to exposed collagen and undergoes a conformational change such that it can bind to platelets. Platelets bind the altered VWF and become tethered to the damaged vessel wall, thus beginning to seal it. Platelets also adhere directly to collagen, but relatively weakly.
3. Platelet aggregation, secretion, and plug formation: Adherent platelets are activated by contact with collagen. Activated platelets secrete numerous stored substances, including ADP, serotonin, Ca^{2+}, polyphosphates, Fbg, VWF, factors V and XIII, and plasminogen, which together help maintain vasoconstriction or promote hemostatic plug formation. Activated platelets also synthesize and secrete TxA2 from arachidonic acid. TxA2 and ADP are platelet activators that recruit more platelets. Activated platelets spread on the surface and undergo membrane changes, including expression of the Fbg receptor, integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa). This promotes platelet aggregation, mostly via interplatelet Fbg bridges (*lower-right blow-up*), thus leading to formation of a relatively fragile primary hemostatic (platelet) plug to patch the defect. Negatively charged inner platelet membrane aminophospholipids (e.g., phosphatidylserine) are externalized on activated platelets and provide phospholipid surfaces for coagulation factors to bind and function.

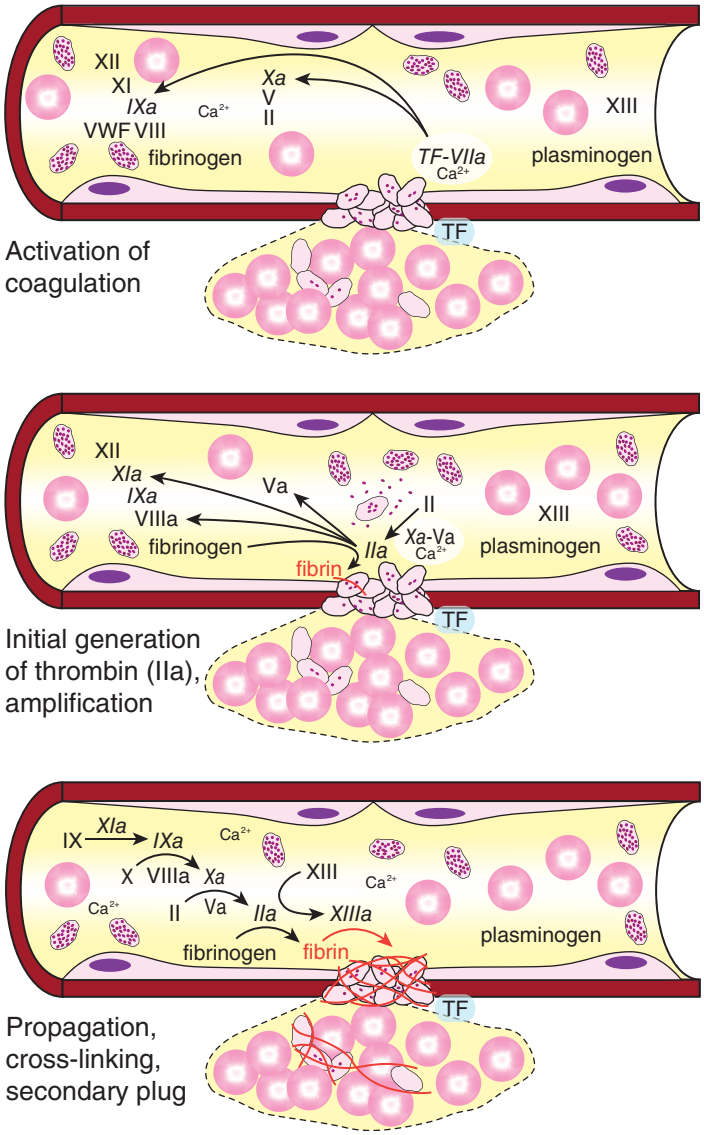


Figure 5.1B The hemostatic process: secondary hemostasis (coagulation).

1. Activation of coagulation: When blood is exposed to TF expressed on cells (e.g., fibroblasts, smooth muscle cells), coagulation is initiated. TF binds to FVII and fCa^{2+}, autoactivating FVII to FVIIa, and VIIa-TF complexes activate factors X and IX of the common and intrinsic pathways, respectively.
2. Initial generation of thrombin and amplification: The resulting FXa binds to FVa and Ca^{2+} on TF-bearing cells, and this prothrombinase complex generates a small amount of thrombin (FIIa) from prothrombin (FII). Concurrent activation of FXII by via collagen occurs but is not shown and is not necessary. The initial small amount of thrombin generated from the TF pathway activates endothelial cells and platelets, recruiting more platelets to the platelet plug. Thrombin activates FXI of the intrinsic pathway and cofactors V and VIII of the common and intrinsic pathways, respectively, initiating amplification of procoagulant activity.
3. Propagation: FIXa formed from FXIa (or TF-VIIa) forms a tenase (X-ase) complex with FVIIIa (and Ca^{2+}) on platelet membranes and activates FX to FXa, which in turn forms prothrombinase complexes with FVa (and Ca^{2+}) to generate thrombin. Thrombin activates more factors and cofactors in an amplification loop that propagates thrombogenesis. It also cleaves Fbg to form fibrin and activates FXIII, which, in the presence of Ca^{2+}, crosslinks fibrin monomers to form polymers that reinforce the primary hemostatic plug and convert it into a stable secondary hemostatic plug that controls bleeding. Some RBCs and WBCs (not shown) are incorporated into the thrombus.

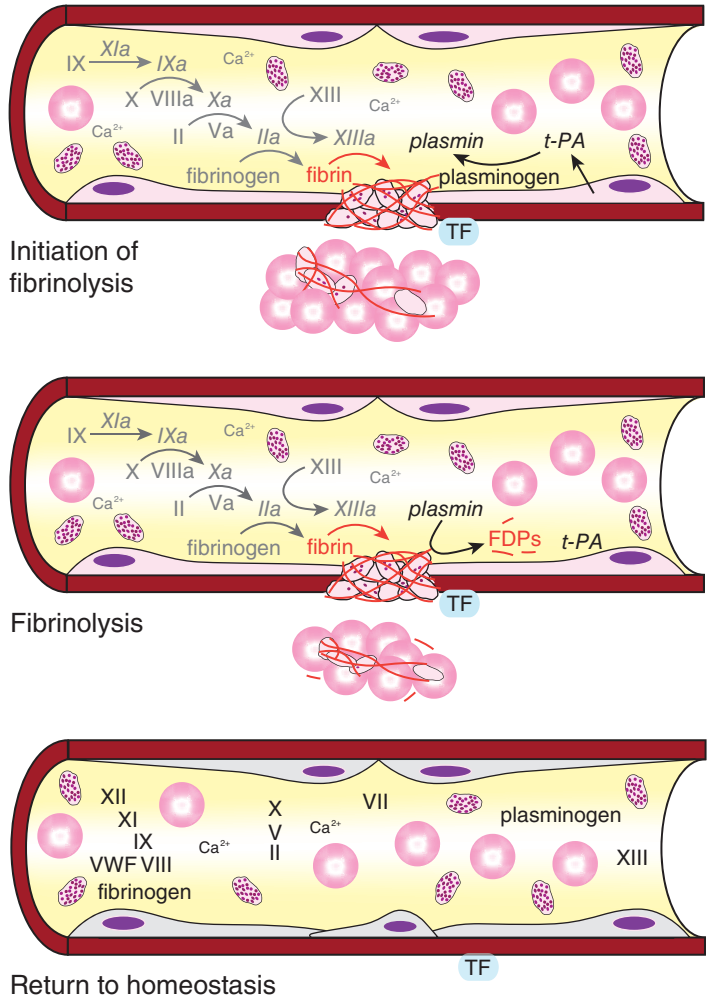


Figure 5.1C The hemostatic process: tertiary hemostasis (fibrinolysis).

1. Initiation of fibrinolysis: The fibrinolytic pathway is activated concurrently with vessel injury and coagulation. Circulating plasminogen binds to fibrin in the hemostatic plug and t-PA is secreted from activated endothelial cells. Thrombus-bound plasminogen is enzymatically cleaved by t-PA to form plasmin.
2. Fibrinolysis: Fibrin is proteolytically cleaved by plasmin to form FDPs. The FDPs include D-dimers because of the cross-linking action of FXIIIa. FDPs have antiplatelet and anticoagulant properties, so fibrinolysis helps controls the extent of coagulation. Tissue u-PA (not shown) also generates plasmin for fibrin degradation.
3. Return to homeostasis: Once fibrinolysis has promoted removal of the hemostatic plug and endothelial cells have resealed the vessel to form a nonthrombogenic barrier, the vessel is repaired.

See the text for information about the role of anticoagulants. Abnormalities of any component of this system can upset the balance and lead to either hemorrhage or thrombosis.

commonly called clots, *clots* are masses of clotted blood, with or without blood cells, that form in vessels after death or when blood leaves vessels, i.e., by hemorrhage or blood collection; blood clots are not attached to a vessel wall.

C. *Tertiary hemostasis* is *fibrinolysis*, the enzymatic degradation of fibrin in a secondary hemostatic plug (Figure 5.1C). Fibrinolysis limits thrombus formation and promotes revascularization.

III. Abnormal hemostasis may cause hemorrhage (bleeding) or thrombosis. Either is a *coagulopathy*, i.e., a disorder of coagulation, but "coagulopathy" is also used to mean specifically a hypocoagulable (bleeding) disorder. Laboratory testing of the individual components of the hemostatic system may be used to discover, explain, monitor, or prognosticate these pathologic states, particularly bleeding disorders. Laboratory tests for detecting thrombosis or early prothrombotic states are limited.

PLATELETS (THROMBOCYTES)

I. Physiologic processes
   A. See Chapter 4 for platelet structure, production, kinetics, and nonhemostatic functions.
   B. Hemostatic functions
      1. Platelets are required for constant maintenance of vascular integrity and formation of primary hemostatic plugs to repair vascular defects. When activated, they help amplify minute stimuli into explosive production of fibrin to form the more-secure secondary hemostatic plugs.
      2. Platelet hemostatic functions can be divided into five major categories (Figure 5.1A):
         a. Adhesion: Platelets adhere to and spread over (patch) exposed subendothelium or perturbed endothelium, mostly via VWF which binds to subendothelial collagen and to platelet GPIb. They also adhere directly, but more weakly, to the subendothelial collagen via the integrin $\alpha_2\beta_1$ (GPIa/IIa).
         b. Aggregation: When adherence or platelet agonists activate platelet membrane integrin $\alpha_{IIb}\beta_3$, platelets aggregate via Fbg (and some VWF) bridges that bind to $\alpha_{IIb}\beta_3$ on neighboring platelets.
         c. Secretion: Activated platelets release preformed granular contents[1] and synthesize and secrete newly formed mediators that promote hemostasis. These include thromboxane A2 and stored ADP that activate and recruit other platelets.
         d. Facilitation of coagulation: When platelets are stimulated, anionic inner membrane aminophospholipids (mostly phosphatidylserine) move to the outer membrane where they provide localizing attachment sites for coagulation cofactors, enzymes, and zymogens. These phospholipids have been referred to as *platelet factor 3*.[2,3] A subset of platelets expressing phosphatidylserine also express high levels of α-granule–derived proteins that promote hemostasis, and these so-called *coated platelets* shed procoagulant microparticles and become incorporated into the platelet aggregate where they support secondary hemostasis. Without secondary hemostasis, the primary plug could become dislodged, causing delayed bleeding.

e. Clot retraction: Platelets within a thrombus facilitate wound closure and vessel patency by a contractile process involving activated platelets, interplatelet bridging via the Fbg receptor $\alpha_{IIb}\beta_3$, and platelet actin and myosin.

3. Mediators released from platelets during the hemostatic process contribute to local tissue repair.

II. Platelet concentration (often called platelet count in clinical jargon, but platelet counts are used to calculate platelet concentrations)

A. See Chapter 4 for analytical concepts and disorders associated with thrombocytopenia and thrombocytosis.

B. Severe thrombocytopenia may cause mucocutaneous hemorrhage (e.g., petechiae, ecchymoses, and epistaxis) or hematomas secondary to vascular trauma (e.g., surgery, venipuncture). Hemorrhage from thrombocytopenia alone is not expected with a [platelet] above ~ $30 \times 10^3/\mu L$ and may not occur with a [platelet] < $5 \times 10^3/\mu L$ in dogs and cats.

1. Hemorrhage may occur at a greater [platelet] if there is a concurrent platelet functional deficit, increased vascular trauma, impaired coagulation, or increased fibrinolysis.
2. Plateletcrit (see Chapter 4) may be a better reflection of platelet hemostatic potential than is [platelet] because large platelets typically have greater hemostatic potential. However, numeric decision limits for using plateletcrit as an indicator of bleeding tendencies were not found.

C. Reactive thrombocytosis is generally not associated with thromboembolic disease or hemorrhagic complications in people. Although thromboembolic disease occurs in a small percentage of dogs with thrombocytosis, concurrent predisposing factors may explain these findings; no cause-and-effect relationship has been shown.[4] Thrombocytosis may yield VET results indicative of in vitro hypercoagulability, but an in vivo prothrombotic state may not be present.[5]

D. Essential thrombocythemia, a myeloproliferative neoplasm, is associated with thrombosis and hemorrhage in people,[6,7] but the diagnosis is rare in domestic mammals, and such associations have not been made in veterinary medicine.

1. Thrombosis associated with human clonal thrombocythemic disorders is not predictable based solely on the [platelet]. Advancing age, previous thrombi, and *JAK2V617F* mutations are risk factors. Mechanisms are unclear, but roles for platelet activation, increased expression of TF, altered endothelial cells function, and increased [RBC] have been proposed.
2. Hemorrhage in human patients with essential thrombocythemia may relate to altered platelet function or acquired VWD.[7]

III. Platelet function tests

A. BMBT and TBT: These are standardized in vivo tests of primary hemostasis. However, there is substantial inter-individual variation and experience is required. The oral mucosa (BMBT test) is used in dogs and cats,[8] whereas the antebrachium (TBT test) is typically used in horses.[9,10] A similar test for capillary bleeding time in the distal limb has been

described for dogs.[11] Although reproducibility is a problem, these tests can detect defects of primary hemostasis. In contrast, cuticle bleeding times obtained by transection of a toenail assess primary plus secondary hemostasis.

1. Analytical concepts
   a. Units are not standardized. Results are sometimes reported in seconds (e.g., 202 s), minutes and seconds (e.g., 3 min 22 s), or minutes (e.g., 3, 3.4, or 3.37 min). Inter-observer and intra-observer imprecision[10,12,13] suggest it would be most appropriate to report to the nearest minute, and an incremental change of ~2 min may be needed between serial BMBTs to conclude an actual change in primary hemostasis. The BMBT of healthy dogs has been reported to range from 1 to 5 min, but it is usually < 4 min.[8,12] The BMBT of sedated cats typically is < 3 min.[13] In horses, mean template bleeding times have varied from about 4 min to 11 min with a maximum of ~15 min.[10]
   b. BMBT procedure: The upper lip is rolled out and usually secured with a gauze strip around the maxilla. Timing begins when a standardized cut (or duplicate cuts) is made in the mucosal surface of the upper lip by using a spring-loaded device (e.g., Surgicutt devices). The cut is small enough (5 mm × 1 mm) for primary hemostasis alone to resolve the bleeding; coagulation and generation of fibrin are not necessary. Filter paper is used to blot away excess blood without touching or disturbing the incision itself. The end point is when bleeding ceases and a crescent of blood no longer forms on the filter paper.
   c. Equine TBT procedure: Authors have described shaving hair caudally just distal to the accessory carpal bone[9] or in the middle portion of the forelimb a few centimeters proximal to the chestnuts,[10] applying a blood pressure cuff proximal to the carpus or sampling area, inflating to 40 mmHg for 1 min, and then discharging the device to produce longitudinal incisions where no superficial vessels are apparent. Blood is gently blotted away with filter paper every 30 s, avoiding the incision, and the template bleeding time is the time from incision to cessation of bleeding.
2. Prolonged BMBT or TBT: These tests are relatively insensitive but are prolonged by moderate to marked defects of primary hemostasis.
   a. Thrombocytopenia (Table 4.2): Marked thrombocytopenia is a contraindication to testing because it is already known that the bleeding time will be prolonged. The degree of thrombocytopenia required to prolong the BMBT or TBT is not clear, but it is commonly stated that BMBT prolongations may occur with platelet concentrations of < 70–100 × 10^3/μL. This decision threshold varies with other factors, such as mean platelet volume (larger platelets may be more functional), [VWF], and Hct (anemia prolongs BMBT).
   b. Impaired platelet function (Tables 5.2 and 5.3): Terms used to describe a disorder of abnormal platelet function include *thrombopathia*, *thrombopathy*, *thrombocytopathy*, and *thrombocytopathia*. Although these are general terms, some have become associated with specific clinical disorders. A platelet functional defect should be suspected if BMBT or TBT is prolonged and platelet concentrations, Hct, and [VWF] are WRI.

(1) Hereditary disorders of platelet function are uncommon (Table 5.2). Several, including causative mutations, have been identified in domestic mammals, but many others identified in people have not yet been identified in domestic mammals. Additional reading is available.[14,15]

(2) Acquired disorders of platelet function (Table 5.3) occur concurrently with disorders or conditions that are usually diagnosed by other findings. The platelet dysfunction may be subclinical, or it may contribute obviously to morbidity.

Table 5.2. Hereditary disorders of intrinsic platelet function

ADP receptor defect – ↓ platelet activation by ADP
$P2Y_{12}$ defect, heterozygotes and homozygotes (greater Swiss mountain dogs)
Fbg receptor ($\alpha_{IIb}\beta_3$) defect – ↓ Fbg binding, ↓ aggregation, ↓ clot retraction
Glanzmann's thrombasthenia, ↓ or defective integrin (great Pyrenees, otterhounds, golden retriever, and mixed-breed dogs; thoroughbred, Oldenbourg, Peruvian paso, and quarter horses); a domestic shorthair cat
Leukocyte adhesion deficiency III – a kindlin 3 defect decreasing integrin activation in platelets and WBCs (a German shepherd and a mixed-breed dog)
VWF receptor (GPIb/IX/V complex) defect
Bernard–Soulier syndrome – ↓ VWF binding, macrothrombocytopenia, ↓ primary hemostasis (European cocker spaniels)
Granule disorders – abnormal granule content or secretion; impaired aggregation
Chédiak–Higashi syndrome – granule dysgenesis in platelets, WBCs, and melanocytes (blue-smoke Persian cats, Hereford cattle, Brangus cattle, and Japanese black cattle)
Cyclic hematopoiesis – a stem cell defect affecting platelets and WBCs (grey collies, their crosses, and rare mixed-breed dogs)
Dense-granule storage-pool disease of American cocker spaniels
Procoagulant expression defects
Scott syndrome – defect in transmembrane protein 16F causing ↓ membrane phosphatidylserine exposure and ↓ microvesiculation (German shepherds)
Signal transduction defect
CalDAG-GEFI thrombopathia – ↓ GTPase activation impairing conformational change in $\alpha_{IIb}\beta_3$ and thus causing ↓ Fbg binding and ↓ aggregation (basset hounds, Eskimo Spitz dogs, European continental type Landseer, and Simmental cattle)
Atypical equine thrombasthenia – possible defect in protein kinase B pathway causing ↓ Fbg binding and ↓ factor V secretion from α granules (thoroughbreds)

CalDAG-GEFI, Ca^{2+} diacylglycerol guanine nucleotide exchange factor I

Note: A platelet functional defect should be considered when (i) petechiae, ecchymoses, mucosal hemorrhages, or trauma-induced bleeding occurs without marked thrombocytopenia, or (ii) bleeding time or clot retraction is prolonged without thrombocytopenia (also consider VWD). However, Scott syndrome causes a coagulopathic pattern of hemorrhage: postoperative bleeding, epistaxis, hematomas, bruising, hemarthrosis, and bleeding with tooth eruption.

Table 5.3. Acquired disorders of intrinsic platelet function
Drug exposure:* some anesthetics (barbiturates), anti-inflammatories (nonsteroidal anti-inflammatory drugs), membrane-active drugs (local anesthetics, β-adrenergic blockers, antihistamines), β-lactam antibiotics (penicillin and cephalosporins), ADP-receptor inhibitors (ticlopidine, clopidogrel), dietary factors (garlic, ethanol, caffeine)
Renal failure (uremia)*
Increased FDPs (increased fibrinolysis, DIC)*
Envenomation (certain venoms)
Hepatic disease
Hyperglobulinemia (plasma cell myeloma, ehrlichiosis)
Immune-mediated thrombocytopenia (only certain antiplatelet antibodies)
Infections: bovine virus diarrhea virus, feline leukemia virus
Neoplasms involving megakaryocytes: myeloproliferative neoplasms, acute megakaryoblastic leukemia
Synthetic colloids (dextran, hetastarch)

* A relatively common disorder, condition, or mechanism

Note: A platelet functional defect should be considered when (i) petechiae, ecchymoses, mucosal hemorrhages, or trauma-induced bleeding occurs without marked thrombocytopenia, or (ii) bleeding time or clot retraction is prolonged without thrombocytopenia (also consider VWD).

c. VWD: The BMBT can be used as a screening test for VWD in breeds predisposed to the disease (e.g., Doberman pinschers). BMBT should be prolonged with VWF:Ag < 20 % and may be prolonged with greater values.
d. Anemia: This may prolong BMBT because the lower [RBC] reduces the number of RBCs in the axial flow, thus forcing fewer platelets to circulate near the vessel wall where they can readily interact with it. Also, fewer RBCs generate less ADP, a platelet agonist.[16,17]
e. Vascular disease: BMBT rarely may be prolonged with certain vascular disorders such as a vasculitis or collagen disorder.
f. Antiplatelet drugs: Aspirin increased forelimb cutaneous bleeding times in horses[9] and also increased canine BMBTs, although the latter were still WRI. Other antiplatelet drugs often do not prolong the BMBT.[18–20]
g. Afibrinogenemia: BMBT may be prolonged in patients with afibrinogenemia, presumably because of the lack of Fbg for interplatelet bridging.[21]

3. Defects restricted to secondary hemostasis and the formation of fibrin (coagulopathies) other than afibrinogenemia should not prolong BMBT unless a larger vessel is cut.[22]
4. Bleeding time is not useful as a screening test for predicting the occurrence of excessive surgical bleeding in human patients.[19]

B. Clot retraction: Because clot retraction in vivo and in vitro is mediated by platelets, some disorders of platelet function (e.g., Glanzmann's thrombasthenia) (Table 5.2) or marked thrombocytopenia (Table 4.2) prolong clot retraction. This may be noted by a decreased yield of serum after 60–90 min of clotting but is more reliably determined by standardized tests of clot retraction:[23]
1. Blood (0.5 mL) is collected into cold saline (4.5 mL) and maintained at 4 °C until transferring to a glass tube and mixing with thrombin (10 U/mL final).

2. The tubes are then placed in a 37 °C water bath. At 1 h, 2 h, and 4 h, clot retraction is graded 1+ (the least retraction) through 4+ (the most retraction) based on the size of the clot and the volume of surrounding serum.
3. Testing should not be done if a patient is thrombocytopenic or has recently received aspirin or other cyclooxygenase inhibitors.
4. Clot retraction is not impaired in patients with VWD.

C. PFA-100 and 200:[24–28] These table-top platelet function analyzers assess the adhesion and aggregation of platelets in 0.8 mL of citrated whole blood by simulating a vascular defect. Within 4 h of collection, blood is aspirated through disposable cartridges and passes at high flow rate through a fine aperture within a membrane coated with various reagents including platelet agonists: collagen and ADP (CADP cartridges), collagen and epinephrine (CEPI cartridges), or ADP with prostaglandin E1, an inhibitor of the adenylate cyclase pathway that is activated when ADP binds to its receptor $P2Y_{12}$ (P2Y cartridges). The agonists and high shear rate induce platelet adhesion via platelet GP1b and VWF, platelet activation, and platelet aggregation via platelet $\alpha_{IIb}\beta_3$ and mostly Fbg. The time (seconds) required until cessation of flow through the aperture is the *closure time*. Valid prolongations of closure times reflect defects in primary hemostasis but not coagulation. Users should establish their own reference values that reflect their sample collection, citrate concentration, delay to testing, and other processing and testing steps. Sample collection and processing should adhere to principles provided in the Analytical concepts section for Coagulation and Anticoagulation. Note: Membranes coated with collagen and epinephrine (CEPI) have shown less value in dogs, cats, and horses because of marked inter-individual variation and some unmeasurable closure times in healthy animals (> 300 s).
1. Prolonged closure time (CADP cartridges) may be caused by:
   a. Certain severe congenital platelet disorders, particularly those related to adhesion and aggregation, though little testing of these has been done in veterinary medicine;[28] prolongations do not occur with defects in platelet procoagulant expression but did occur in American cocker spaniels with dense granule storage disease
   b. Certain acquired defects in platelet function (many have not been tested), such as those occurring with use of clopidogrel or aspirin;[25,27,29] antiplatelet drugs may induce prolongations from baseline without exceeding the URL
   c. VWD when VWF:Ag is low enough (< ~25 %)[11,30]
   d. Thrombocytopenia with [platelet] < ~100,000/μL, a contraindication for testing[11,28]
   e. Anemia (Hct < ~30 %); closure time progressively increases as Hct decreases from 40 %, thus limiting use of this system in anemic patients[11,28]
   f. Disorders of coagulation such as vitamin K antagonism and hemophilia do not prolong closure times.[11]
2. A reduced closure time might reflect a prothrombotic state, increased VWF activity, or erythrocytosis, but studies have focused on prolongations.
3. No closure time may be provided, e.g., with flow obstruction errors. When assessing effects of clopidogrel on feline platelet function using the PFA-200 and P2Y cartridges, the analyzer's research parameters helped indicate clopidogrel's effects in the absence of reported closure times.[27]

D. Plateletworks: Reagent tubes are commercially available to assess the platelet response to ADP, collagen, or arachidonic acid. The ADP tubes have been used to assess clopidogrel

effects in cats, a minority of which have platelets that are not inhibited by clopidogrel.[31] Blood is carefully drawn into an EDTA tube and a tube containing 3.2 % citrate plus ADP. The [platelet] of each sample is promptly measured.

1. In health, and without substantial collection-induced platelet clumping, the [platelet] of the ADP/citrate sample is lower than that of the EDTA sample because many platelets aggregate and are not counted. The [platelet] in the ADP/citrate-tube is reported as a percentage of the EDTA-tube [platelet] and considered the % aggregation; when few platelets aggregate, the platelet concentrations are similar so the difference in [platelet] is relatively low, and the % aggregation is relatively high. RIs must be determined for a particular hematology analyzer and cat population.
2. Clopidogrel-treated cats with clopidogrel-resistant platelets yield results similar to most healthy cats, with a relatively high % aggregation.
3. Clopidogrel-treated cats with clopidogrel-sensitive platelets yield lower % aggregation results because clopidogrel impairs ADP-induced aggregation.

E. VET (see Global Assessment of Hemostasis)

F. Other specialized tests are used to assess platelet responses in vitro.[24] These tests generally require labs with specialized equipment and species-specific expertise. Because platelets are easily activated during collection and handling, and their function changes within hours of collection, most platelet function studies require on-site equipment and blood collection that follows rigid guidelines aligned with ideal blood collection for coagulation assays (see Coagulation and Anticoagulation, Section II).

1. Flow cytometry can be used to assess membrane glycoproteins that are decreased in certain inherited platelet disorders. Platelets can be assessed for hypoactivity or hyperreactivity via activation markers such as increased P-selectin (CD62P) or bound Fbg. Surface phosphatidylserine expression can be detected to assess procoagulant potential, and there is potential to assess platelet microparticles and their contribution to prothrombotic states. Platelets may also be detected in platelet–WBC aggregates that are markers of thrombo-inflammatory disease.
2. Platelet aggregometry can be used to assess the responsiveness of platelets to various stimuli in platelet-rich plasma or whole blood. Depending on the system, detection of platelet agglutination (passive clumping) or aggregation (active clumping) is based on changes in light transmission or changes in impedance as platelets accumulate on electrodes. Lumiaggregometers can additionally assess platelet secretion of adenosine triphosphate (ATP) from dense granules. Although used mostly as a research tool to investigate platelet hypo- and hyperreactive states or response to drugs, aggregation studies can be used to characterize rare platelet function defects and provide diagnostic support for specific disorders such as CalDAG-GEFI thrombopathia. Species-specific experience is critical.
3. Molecular genetic testing can detect hereditary platelet disorders for which mutations have been characterized.[14]

VON WILLEBRAND FACTOR (VWF)

I. Physiologic processes

A. *VWF* is a large multimeric plasma glycoprotein ($M_r \cong 500{,}000–20{,}000{,}000$) important in platelet adhesion and aggregation. It has several important binding domains, including those for FVIII (which it carries in blood), collagen, and platelet receptors (GP1b and $\alpha_{IIb}\beta_3$).

1. Adhesion: VWF bridges platelets to injured vessel walls via platelet GPIb and exposed subendothelial proteins such as collagen. This binding is particularly important in vessels with higher velocity blood flow and high shear stress; platelet adhesion is also promoted by such environments. Shear stress, which is greatest in arterioles or stenotic vessels, increases as the difference in flow rate between the center and periphery of a vessel increases.
2. Aggregation: VWF contributes to platelet–platelet bridging via GPIb and the platelet integrin $\alpha_{IIb}\beta_3$.
3. The largest multimers of VWF are the most functional.[32]

B. Most VWF is produced by endothelial cells[33,34] and secreted constitutively or stored and secreted later upon endothelial cell activation. Megakaryocytes also synthesize VWF. Megakaryocytes and platelets contain a substantial percentage of total circulating VWF in most species (e.g., cats and people), but canine platelets contain relatively little.[1,35,36]

C. Secreted VWF forms noncovalent complexes with coagulation FVIII and serves as a stabilizing and protective carrier molecule for FVIII.[37]

D. In people, and likely in domestic mammals, ultralarge VWF multimers are rapidly cleaved under high-shear states to smaller, less functional forms by ADAMTS-13, a disintegrin and metalloprotease with thrombospondin type 1 repeats-13 (VWF-cleaving protease, EC 3.4.24.87) produced mostly in the liver. This regulates platelet adhesion and thrombus formation.
1. Deficiencies in functional ADAMTS-13 (e.g., mutations, autoantibody inhibition) cause pathologic platelet–vessel wall interactions and sometimes widespread thrombosis (thrombotic thrombocytopenic purpura) in people.
2. Recombinant canine ADAMTS13 cleaves human VWF, and initial evaluation of an ELISA based on immunodetection of a cleaved human VWF substrate suggests that the human assay detects ADAMTS13 activity in canine plasma.[38] As expected, mean ADAMTS13 activity was decreased in bacteremic dogs compared to healthy dogs. Immunohistochemical detection of ADAMST13 has been reported in equine tissues.

II. von Willebrand disease (VWD)

A. *VWD*, a disorder of primary hemostasis caused by a deficiency in functional VWF, is the most common hereditary bleeding disorder in dogs but is rare in cattle,[39] cats,[40,41] and horses.[15,42–44]

B. VWD is an inherited disorder, whereas von Willebrand syndrome (VWS) is acquired and develops uncommonly in association with neoplastic, cardiovascular, or autoimmune disorders in people[45] and perhaps dogs. Pathogenic mechanisms vary with the cause.
1. One of the diseases associated with human acquired von Willebrand syndrome is hypothyroidism, and treatment for hypothyroidism has resolved the concurrent VWF deficiencies.[46] An association between hypothyroidism and VWD has also been suggested in dogs, particularly in Doberman pinschers.[47] However, studies have produced conflicting results, and the concurrence of common diseases does not prove a cause-and-effect relationship.[32] In one study, VWF:Ag values were WRI for hypothyroid dogs before treatment with levothyroxine and significantly decreased (not increased) after treatment, indicating that there is not a predictable association between hypothyroidism and VWD in dogs.[48]
2. Transient acquired von Willebrand syndrome has been reported in rare dogs infected with *Angiostrongylus vasorum*.[49] One dog had a concurrent transient deficiency of FVIII.

C. Three general types of VWD have been defined (though subtypes exist):
   1. Type 1: All VWF multimers are present but at decreased concentrations. The severity of type 1 VWD varies. It is the most common form and occurs in many dog breeds, including Doberman pinschers.[50] It may occur in horses.[43]
   2. Type 2: This severe, uncommon form is a deficiency of VWF with a disproportionate decrease in large multimers (type 2A). It occurs in German shorthaired pointers and German wirehaired pointers[51] and has been reported in the quarter horse and thoroughbred breeds.[42,44]
   3. Type 3: This severe form, which involves an absence of all VWF multimers, occurs particularly in Chesapeake Bay retrievers, the kooikerhondje, Scottish terriers, Shetland sheepdogs, and mixed breed dogs.[52–55] It has been reported in a domestic long-haired cat.[41]

D. Clinical and laboratory signs of VWD
   1. Mild to severe mucosal hemorrhage (epistaxis, gastrointestinal hemorrhage, or prolonged estral bleeding), hematuria, cutaneous bruising, and prolonged hemorrhage or hematomas from nonsurgical or surgical trauma (e.g., tooth eruption, tail docking, dewclaw removal in puppies, or tooth extraction); hemarthrosis and hematomas in horses
   2. Absence of petechiae help differentiate VWD from platelet disorders.
   3. Prolonged BMBT or TBT without thrombocytopenia and without prolonged coagulation times
   4. PTT may be mildly prolonged because decreased FVIII coagulation activity (FVIII:C) may occur secondary to reductions in circulating VWF, a carrier molecule for FVIII.
      a. However, in contrast to human patients with VWD, FVIII:C in dogs with VWD is usually > 30 % of the activity in reference plasma, so PTT is usually WRI, even in dogs with type 3 VWD (no VWF).[32,52,56]
      b. A 7-yr-old Shetland sheepdog was diagnosed with type III VWD and primary hypothyroidism when it was presented for epistaxis associated with an apparent transient FVIII deficiency; hypothyroidism may have contributed to the coagulopathy.[57]
      c. PTT may be prolonged in horses with VWD.[44]

III. Analytical concepts

A. Sample
   1. Plasma is collected from blood drawn into sodium citrate or EDTA; citrate tubes should be filled to give a 1:9 volume ratio of citrate to blood. The VWF:Ag may be markedly decreased in samples with clots or in vitro hemolysis, but lipemia has no significant effect.[58]
   2. It is generally recommended that plasma be collected promptly after blood collection, frozen, and shipped overnight with ice. The VWF:Ag is reportedly stable for at least 8 h in canine plasma or whole blood stored at room temperature.[58] However, values are significantly increased at 24 h after collection when whole blood samples are stored at room temperature, and values are increased at 48 h (but not 24 h) after collection when plasma is stored at room temperature.[59] These increases did not occur when samples were refrigerated.

B. Units: %, U/dL, or U/mL relative to 100 %, 100 U/dL, or 100 U/mL, respectively, of VWF:Ag in pooled plasma from healthy individuals of the same species. Units may be written to indicate the species of the patient and reference samples, e.g., CU/dL for canine (C) samples. Results can vary with the composition of reference pooled plasma.

C. VWF:Ag assays[32,60–63]

1. ELISA: VWF is usually measured by a quantitative ELISA with species-appropriate polyclonal or monoclonal antibodies to VWF. Values < 50 % are usually considered decreased.
2. Multimeric analysis (immunoelectrophoresis)
   a. VWF multimers are separated by agarose electrophoresis so that the relative amounts of different sized multimers can be determined.
   b. This can differentiate type 1 VWD (high molecular weight multimers present) from type 2A VWD (high molecular weight multimers absent).
3. Functional assays
   a. VWF collagen-binding capacity (VWF:CB) may be measured in canine plasma by an ELISA that detects only the VWF that can bind to immobilized bovine collagen.[30] The assay therefore measures the quantity of functional VWF and preferentially detects the more functional large multimers. The ratio of VWF:Ag to VWF:CB, which can be determined when the assays are used for the same sample, may be increased in type 2 VWD because of a decrease in large multimers and therefore a greater decrease in functional VWF than in VWF antigen. The ratio is close to one in type 1 VWD but greater than two with type 2A VWD.
   b. Ristocetin and botrocetin cofactor activities (VWF:RCo and VWF:BCo, respectively): These aggregometer-based assays are little used in veterinary medicine. Agglutination (passive clumping, not active aggregation) of platelets is measured in the presence of the antimicrobial peptide ristocetin or the nonenzymatic snake venom protein botrocetin. Ristocetin, which is widely used in human assays, has some reactivity with equine VWF but does not react with canine VWF, so botrocetin is used for dogs. Ristocetin and botrocetin mediate the linking of platelets via VWF bridging between GP1b on adjacent platelets. The rate of VWF-dependent platelet agglutination correlates well with VWF:Ag unless large multimers, which are more functional, are disproportionately deficient (type 2A VWD); in that case, cofactor activity may be markedly reduced with little or no decrease in VWF:Ag. VWF:RCo and VWF:BCo are reported as percentages of the agglutination rate or maximal agglutination compared to a species-specific plasma pool.

IV. Decreased VWF Concentration (VWF:Ag) (Table 5.4)

Table 5.4. Disorders and conditions that cause decreased VWF:Ag

VWD: VWF:Ag usually < 35 %
Type I:* all VWF multimers present but proportionately deficient
Type II: deficiency of VWF multimers with high M_r
Type III: absence of all VWF multimers, VWF:Ag ≅ 0 %
VWD carrier (rarely symptomatic): VWF:Ag usually 30–70 %*

* A relatively common disorder or condition (dogs)
Note: The unit for VWF:Ag is the % of the result for plasma pooled from healthy dogs.
Hemolysis or clotted samples may cause marked false decreases in VWF:Ag.

A. Decreased plasma VWF:Ag is indicative of the VWD trait or a carrier state for it, depending on the degree of the decrease. Clinical signs of impaired hemostasis may not be present. The following are common guidelines for ELISA VWF:Ag results in dogs:[64]
   1. Dogs with VWF:Ag values < 50 % (cutoff may vary with assay) are considered carriers of the VWD trait. They are at risk for clinical disease and are likely to transmit the trait to offspring. The risk of clinical VWD is greater at lower VWF:Ag values.
   2. Dogs with borderline VWF:Ag values of 50–69 % (may vary with assay) are at little or no risk for clinical disease but may be carriers that can transmit the trait to offspring. Repeated testing may clarify if they are affected (result < 50 %) or not (result > 69 %).
   3. Dogs with type 3 VWD have essentially no VWF:Ag.
   4. Most dogs that bleed because of VWD have VWF:Ag values < 35 %.

B. Dogs with at least 70 % plasma VWF:Ag (cutoff may vary with assay) are considered free from the VWD trait. They are not at risk for clinical disease and have a very low risk for transmitting the trait to offspring.

C. An increase in the VWF:Ag to VWF:CB ratio is supportive of type 2A VWD. Multimer analysis could also be used to confirm type 2 VWD.

V. Interpretive considerations

A. See the foregoing section (Analytical concepts, Section IIIA) for effects of improper sample processing.

B. Intra-individual biological variation: VWF:Ag has been shown to vary considerably from day to day when healthy or diseased (VWD) dogs are sampled serially.[58]

C. Exercise: VWF:Ag may be increased in dogs after very strenuous exercise.[65–67] The VWF:Ag in horses increased as much as 100 % within 15 min after strenuous exercise, returning to baseline by 30 min before increasing again after 90 min.[68]

D. Pregnancy: Substantial increases occurred in bitches at parturition, with lesser increases in the last trimester of pregnancy and for the first 1–2 wk after parturition.[69,70] Type I VWD could be masked by testing during these times.

E. DDAVP (1-deamino-8-D-arginine vasopressin):[65]
   1. DDAVP increases the plasma VWF:Ag in dogs with type 1 VWD or in dogs without VWD by releasing VWF from endothelial cell stores.[71] Improved hemostasis after DDAVP may not be due to preferential release of larger multimers as in people. In one study, the VWF:CB increased proportionally to the VWF:Ag after DDAVP administration to dogs, without a disproportionate increase in large multimers.[63]
   2. DDAVP can be used effectively to increase the amount of VWF in blood donor animals when administered 30–90 min prior to blood collection.[72]
   3. In people, DDAVP also promotes primary hemostasis by enhancing formation of procoagulant platelets and platelet-dependent thrombin generation via increased [Na^+] and [Ca^{2+}] within platelets.[73]

F. VWF:Ag may also be increased by epinephrine,[65] endotoxin,[74] azotemia,[75] feline hyperthyroidism,[76] some hepatic diseases,[77] and other illnesses.

VI. Genetic tests have been developed that can detect some VWF gene mutations in several breeds of dogs and may be useful to detect carriers in specific breeds.[55,78,79]

COAGULATION AND ANTICOAGULATION

I. Physiologic processes (Figure 5.1B)
   A. Coagulation is only one component of hemostasis, and although intimately associated with endothelial cells, platelets, the fibrinolytic system, and other blood cells, it is distinct from these components and can be evaluated independently in vitro.
   B. Coagulation involves an interconnected series of cell-associated enzyme-activating steps that form thrombin (FIIa) and convert soluble Fbg (FI) into insoluble fibrin within a secondary hemostatic plug. Most steps involve an enzyme, a substrate (Fbg, fibrin, or proenzyme forms of coagulation enzymes), and a cofactor (e.g., FVa or VIIIa) assembled and localized on a phospholipid surface (e.g., platelet, WBC, or endothelial cell membranes) in the presence of fCa^{2+}.
   C. The coagulation cascade or web (Figure 5.2) can be divided into three pathways that are bridged together in vivo but assessed separately in vitro. The TF (extrinsic) and surface-induced (intrinsic) pathways lead to the common pathway that generates thrombin, an enzyme that converts Fbg to fibrin to form a thrombus or clot.
      1. TF (extrinsic) pathway: This pathway is initiated by TF, which is expressed in active form (does not need activation) on vascular smooth muscle cells, activated monocytes, fibroblasts, other extravascular cells, stimulated endothelial cells, or microparticles released from these cells. It leads to activation of FX at the start of the common pathway. TFPI, as its name indicates, inactivates the TF-VIIa complex, but not until thrombin is generated through the common pathway.
      2. Surface-induced (intrinsic) pathway: This pathway is activated secondary to activation of the TF pathway, but it also can be activated directly.
         a. Secondary activation:
            (1) TF-VIIa activates FIX, thus bypassing inhibition by TFPI.
            (2) When a small amount of thrombin is generated from the extrinsic plus common pathways, it activates factors XI and VIII in the surface-induced pathway and FV in the common pathway leading to markedly amplified thrombin generation.
         b. Direct activation: The surface-induced (intrinsic) pathway can be initiated when the contact coagulation factors HK, PK, and FXII contact negatively charged surfaces such as collagen, DNA (e.g., from neutrophil extracellular traps), or polyphosphates from microorganisms or platelet dense granules[80] and form FXIIa. FXIIa generates kallikrein which generates more FXIIa in a contact pathway amplification loop that also generates the proinflammatory mediator bradykinin. This intersection of inflammation and coagulation can mediate immunothrombosis, the generation of infection-controlling microthrombi via contact activation by the innate immune system.[81] The surface-induced pathway is inhibited by AT, protein C, and protein S.
      3. Common pathway (common continuation of surface-induced and TF pathways): This pathway is initiated by activation of FX to FXa by either the intrinsic-pathway tenase (X-ase) or TF-pathway tenase complexes. It leads to formation of thrombin (IIa) from prothrombin by the prothrombinase complex.
         a. Prothrombotic thrombin effects:
            (1) By activating factors XI, VIII, and V, thrombin amplifies its own generation through the surface-induced (intrinsic) and then common pathways.

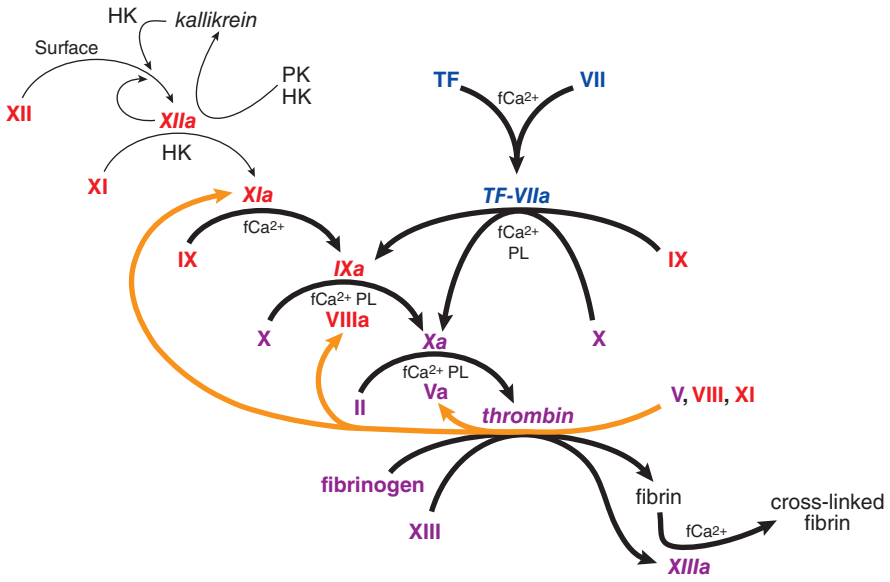


Figure 5.2. Coagulation cascade. The coagulation cascade begins with TF in the TF (extrinsic) pathway or with activation of FXII of the surface-induced (intrinsic) pathway, either pathway resulting in the formation of cross-linked fibrin by the common pathway which begins with FX. *Bold arrows* represent major coagulation pathways in vivo as illustrated in Figure 5.1.

- TF (extrinsic) pathway (*blue factors*): Consisting only of TF and FVII, this short pathway requires addition of something *extrinsic* to blood (TF) for in vitro activation. Although extravascular TF is a major initiator, TF can also be expressed on circulating monocytes, endothelial cells, and microparticles activated by endotoxin and certain inflammatory cytokines, so activation is not necessarily extrinsic to the blood. The TF pathway activates the common pathway (X) and, in vivo, also activates the surface-induced pathway via factor IX.
- Surface-induced (intrinsic) pathway (*red factors*): Beginning with FXII, this longer pathway was named *intrinsic* because it mediated clotting in glass tubes with no added factor, although the glass itself was the contact-activating factor. Activation occurs when FXII contacts a negatively charged surface such as subendothelial collagen exposed at the site of vascular injury. However, surface activation plays a minor hemostatic role; patients with single contact-factor deficiencies (FXII, HK, or PK) do not have clinical bleeding disorders. In vitro, kaolin, silica, ellagic acid, diatomaceous earth, or glass surfaces are activators.
  - Surface-activated FXIIa activates itself (autoactivation). Surface-bound FXIIa, in the presence of HK, activates PK to kallikrein, which enzymatically produces more FXIIa in a potent amplification loop called the contact pathway.
  - FXIIa, again with HK, cleaves FXI to yield FXIa, which cleaves factor IX in the presence of fCa^{2+} to form FIXa. FIXa then binds to the PL surface (requires fCa^{2+}) to form the tenase complex with FVIIIa, activated mostly by thrombin (FIIa).
- Common pathway (*purple factors*): This is the common continuation of the TF and surface-induced pathways, beginning with FX, which is cleaved to FXa. FXa complexes with FVa (activated mostly by thrombin) and fCa^{2+} on PL surfaces to form *prothrombinase complexes*, which enzymatically convert prothrombin (II) to thrombin (IIa). Thrombin then activates platelets and endothelial cells, and cleaves many substrates, including the following:
  - Fbg: Fibrinopeptides A and B are cleaved from Fbg to form fibrin monomers, which polymerize into fibrin polymers (Figure 5.7).
  - FXIII: Proteolytic cleavage of FXIII leads to activation. FXIIIa, in the presence of fCa^{2+}, cross-links fibrin and reinforces the secondary hemostatic plug.
  - FXI: Proteolytic activation of FXI along with pro-cofactors V and VIII provides positive feedback and marked amplification of coagulation through the intrinsic and common pathways (*gold arrows*). In this way, the small amounts of thrombin initially formed from TF pathway activation translate into large amounts of thrombin.

(2) By cleaving Fbg to fibrin monomers that form multimers, thrombin creates the insoluble matrix of the secondary hemostatic plug.
(3) Through activation of FXIII, the fibrin multimers are cross-linked to strengthen the thrombus and limit its degradation. FXIIIa also cross-links fibrin to α_2-antiplasmin which then inhibits fibrinolysis within the thrombus. FXIIIa and platelets promote clot retraction; the proenzyme subunit of FXIII is conveniently present in platelets as well as plasma.
(4) Thrombin also is a potent platelet activator, causing platelet secretion, aggregation, and procoagulant expression.

b. Antithrombotic thrombin effects:
(1) Thrombin binds to thrombomodulin on endothelial cells, and the complex activates circulating protein C to APC. APC (with protein S) inactivates the cofactors Va and VIIIa; it also promotes fibrinolysis via t-PA. Antiproteases quickly clear t-PA, which has a half-life of minutes.
(2) Thrombin bound to thrombomodulin also activates TAFI, and TAFIa inhibits fibrinolysis, primarily by cleaving plasminogen-binding sites from fibrin.

c. In addition to inhibition by proteins C and S, the common pathway is inhibited by AT.

D. Coagulation factors (Table 5.5)
1. Enzymatic coagulation factors circulate as inactive proenzymes (zymogens) until they are activated.
a. They are produced primarily in hepatocytes, but FVIII appears to be produced in endothelial cells of hepatic sinusoids. The production of factors II, VII, IX, and X is vitamin K dependent (Figure 5.3). The "K" in vitamin K is derived from the German *Koagulations Vitamin* (i.e., "coagulation vitamin").[82]
b. FIX is sex linked. Its gene is on the X chromosome, whereas genes for the other coagulation enzymes are autosomal.[83,84]
c. Half-lives of proenzymes in health: In people, half-lives range from a few hours (FVII) to several days (FII and FXIII), with most being about 1–2 d.[85] Half-lives of coagulation factors in domestic mammals are presumed to be similar. The half-life of FVII was < 5 h in FVII-deficient beagles transfused with plasma from healthy dogs.[85]
d. Most enzymatic factors are not directly destroyed during coagulation (they are present in serum). Instead, the activated factors are complexed by inhibitors, and the complexes are cleared by hepatocytes or the MPS. Enzymatic degradation of some enzymatic factors (e.g., FXIII by plasmin) does occur. Some thrombin bound to thrombomodulin on endothelial cells is internalized and destroyed by endothelial cells.

2. Nonenzymatic coagulation factors are TF, Fbg, procofactors V and VIII, fCa^{2+}, and phospholipid.
a. *TF* (CD142) is the transmembrane glycoprotein receptor and cofactor for factors VII and VIIa, and the major activator of coagulation in vivo. It can mediate cell signaling and appears to participate in several nonhemostatic processes (e.g., angiogenesis and metastasis).

Table 5.5. Coagulation factors, abbreviations, and roles

Factor	Name	Pathway	Function
I	Fibrinogen	Common	Substrate for thrombin; converted to fibrin
II	Prothrombin	Common	Proenzyme of thrombin which cleaves Fbg and activates FV, FVIII, FXI, FXIII, protein C, and platelets
(III)[a]	TF, tissue thromboplastin	TF	Cofactor: TF binds and activates FVII, and the TF/FVIIa complex activates FIX and FX
(IV)	fCa^{2+}	All	Cofactor for factors IIa, VIIa, IXa, Xa, and XIIIa
V	Proaccelerin	Common	Pro-cofactor for FXa; cofactor after activation to FVa
VII	Proconvertin, stable factor	TF	Proenzyme of FVIIa which activates FIX and FX
VIII	Antihemophilic factor	Surface-induced	Pro-cofactor for FIXa; cofactor after activation to FVIIIa
IX	Christmas factor	Surface-induced	Proenzyme of FIXa which activates FX
X	Stuart factor, Stuart–Prower factor	Common	Proenzyme of FXa which activates FII
XI	Plasma thromboplastin antecedent	Surface-induced	Proenzyme of FXIa which activates FIX
XII	Hageman factor	Surface-induced	Proenzyme of XIIa which activates FXI, PK, HK, and plasminogen
XIII	Fibrin-stabilizing factor, fibrinase	Common	Proenzyme of FXIIIa which cross-links fibrin and protects it from plasmin degradation; promotes clot retraction
HK	Fitzgerald factor	Surface-induced	Cofactor for activation of FXII and FXI
PF3	Platelet factor 3, mostly phosphatidylserine	Surface-induced, common	Negatively charged platelet membrane phospholipids important for in vivo activation of FX and FII
PK	Fletcher factor	Surface-induced	Proenzyme of kallikrein; activates FXII and PK, generates bradykinin from HK, promotes plasmin generation

[a] Parentheses indicate that the Roman numeral is rarely used.

Note: Roman numeral VI is unassigned; a proposed factor VI was later identified as factor Va. The TF pathway is also called the extrinsic pathway; the surface-induced pathway is also called the intrinsic pathway or contact pathway.

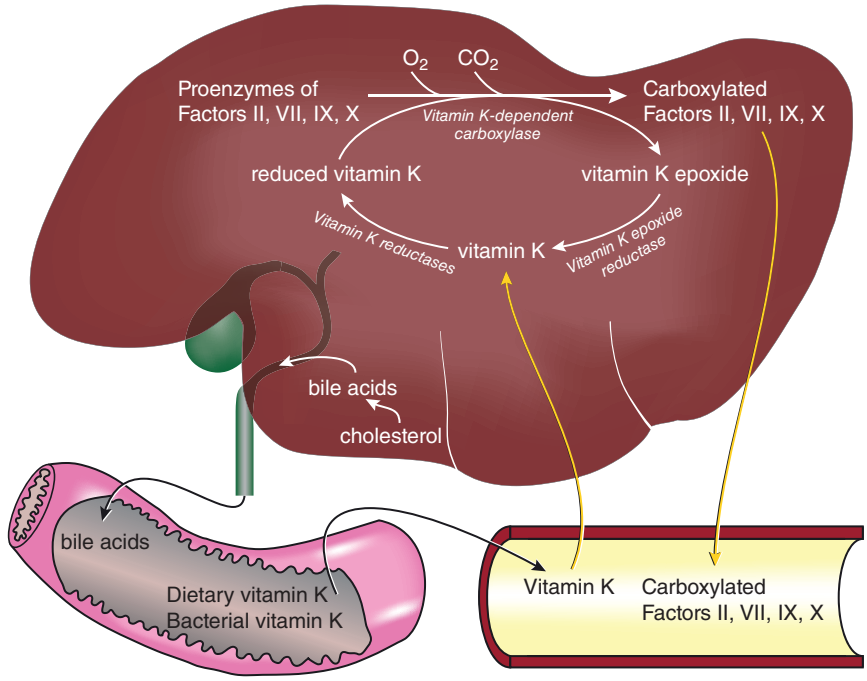


Figure 5.3. Synthesis of vitamin K–dependent factors. Coagulation factors II (prothrombin), VII, IX, and X are synthesized primarily in hepatocytes. Vitamin K is required for these factors to be functional, i.e., they are vitamin K dependent (as are the anticoagulants protein C and S).

- Vitamin K is ingested and also produced by intestinal bacteria. As a fat-soluble vitamin, it is absorbed with lipid that is digested by lipase and emulsified by bile acids.
- In hepatocytes, vitamin K is reduced to its active form (reduced vitamin K). Reduced vitamin K is a cofactor for a vitamin K–dependent γ-glutamyl carboxylase, the enzyme responsible for posttranslational γ-carboxylation of glutamic acid residues in these factors. Carboxylation is needed so that the factors can bind fCa^{2+}, which induces conformational changes and enables binding to phospholipid membranes that are needed for coagulation.
- Reduced vitamin K becomes oxidized to vitamin K epoxide during carboxylation, requiring enzymatic reduction before it can again function as a cofactor for vitamin K–dependent carboxylase. Reduction of vitamin K epoxide to vitamin K and then of vitamin K to reduced vitamin K (vitamin K hydroquinone) is mediated primarily by vitamin K epoxide reductase (vitamin K oxidoreductase C1), a warfarin-sensitive enzyme, but at least one other reductase can participate in the second reductive step.
- Carboxylated factors II, VII, IX, and X enter the blood, where they can be activated to participate in enzymatic reactions of the coagulation system.

b. *Fbg* is a positive acute-phase protein produced by hepatocytes. It has a half-life of 2–3 d in healthy dogs[86] and 4–5 d in healthy horses,[87] but its catabolism may be increased with acidemia.[88] It is consumed during coagulation as it is converted to fibrin by thrombin.

c. Cofactors V and VIII markedly accelerate coagulation by facilitating surface attachment and localization of coagulation factors of the intrinsic and common pathways.

(1) FV is thought to be synthesized mainly in hepatocytes, but some may be produced in other cells including megakaryocytes. It has a half-life of about 0.5–1.5 d and is consumed by APC during coagulation.

(2) FVIII synthesis appears to occur primarily in endothelial cells of hepatic sinusoids. Like FIX, it is sex linked; its gene is on the X chromosome.[89] It circulates in a noncovalent complex with VWF but is distinct from VWF. The half-life of human FVIII is about 0.5 d, but it is less in the absence of VWF. Like FVa, FVIIIa is consumed by APC during coagulation and is not present in serum. It is a positive acute-phase protein, increasing in plasma with exercise and inflammation.[90,91]

d. EDTA, oxalate, and citrate function as in vitro anticoagulants by binding fCa^{2+} and preventing it from interacting with coagulation proteins. In vivo, there is always enough fCa^{2+} for coagulation even with hypocalcemia.

E. Anticoagulants: Physiologic inhibitors of coagulation help prevent excessive coagulation.[92] Deficiencies of these anticoagulants are associated with thromboembolic disease.

1. *AT* (also called *ATIII*), a protein ($M_r \cong 58{,}000$) produced primarily by hepatocytes, inhibits all the coagulation enzymes in the surface-induced, TF, and common pathways. Despite what its name might imply, its function is not only against thrombin. It binds, inactivates, and removes all the coagulation enzymes from the circulation, most importantly thrombin (FIIa), FIXa, and FXa. AT–enzyme complexes are rapidly cleared via receptors on hepatocytes.[93] AT activity is markedly enhanced by exogenous heparin, endogenous heparin from neoplastic mast cells or nonneoplastic mast cells in anaphylaxis, and heparan sulfate, a major component of the endothelial glycocalyx (Figure 5.4).
2. *Protein C*, a vitamin K–dependent proenzyme anticoagulant and profibrinolytic agent ($M_r \cong 62{,}000$), inhibits coagulation in the surface-induced (intrinsic) and common pathways. The *C* stands for the third (A, B, C) fraction eluted from a column.[94] It is produced in hepatocytes and circulates in plasma with a half-life of 6–10 h in people. It should not be confused with C-reactive protein, a positive acute-phase protein.
   a. Protein C is activated to APC on endothelial cells by a complex of thrombin bound to thrombomodulin, a thrombin receptor, and endothelial protein-C receptor (EPCR). APC then inactivates cofactors Va and VIIIa by proteolytic cleavage in the presence of FV[95] and membrane-bound protein S. This greatly reduces thrombin generation.
   b. Less thrombin to activate TAFI promotes fibrinolysis, and APC promotes fibrinolysis through inhibition of PAI-1. APC also has important anti-inflammatory and cytoprotective effects.
   c. *Protein S* is a vitamin K–dependent, nonenzymatic cofactor produced by endothelial cells, hepatocytes, and megakaryocytes. The *S* stands for "Seattle," where the protein was first described.[94] It circulates free and bound to complement 4b-binding protein; the complement-bound form has reduced activity. Protein S localizes processes to cell membranes by simultaneously binding to phospholipid membranes and various proteins. In addition to its APC-dependent anticoagulant effects, protein S promotes inhibition of FXa by TFPI, and it may directly inhibit cofactors Va and VIIIa.
3. *TFPI* ($M_r \cong 40{,}000$) quickly inhibits coagulation induced by the TF pathway. There are no known deficiencies of TFPI, and it appears to be necessary for life. It is produced primarily by endothelial cells; other TFPI-producing cells include monocytes, macrophages, and vascular smooth muscle cells. Most TFPI is associated with vessel walls, but it also circulates in platelets and in plasma, much of it bound to lipoproteins. Heparin promotes endothelial cell release of TFPI. With initiation of coagulation by the TF pathway, TFPI inhibits TF-FVIIa by forming a stable quaternary complex in the presence of fCa^{2+}: TF-FVIIa–FXa–TFPI.[96] This inhibits further generation of FXa via TF-FVIIa.

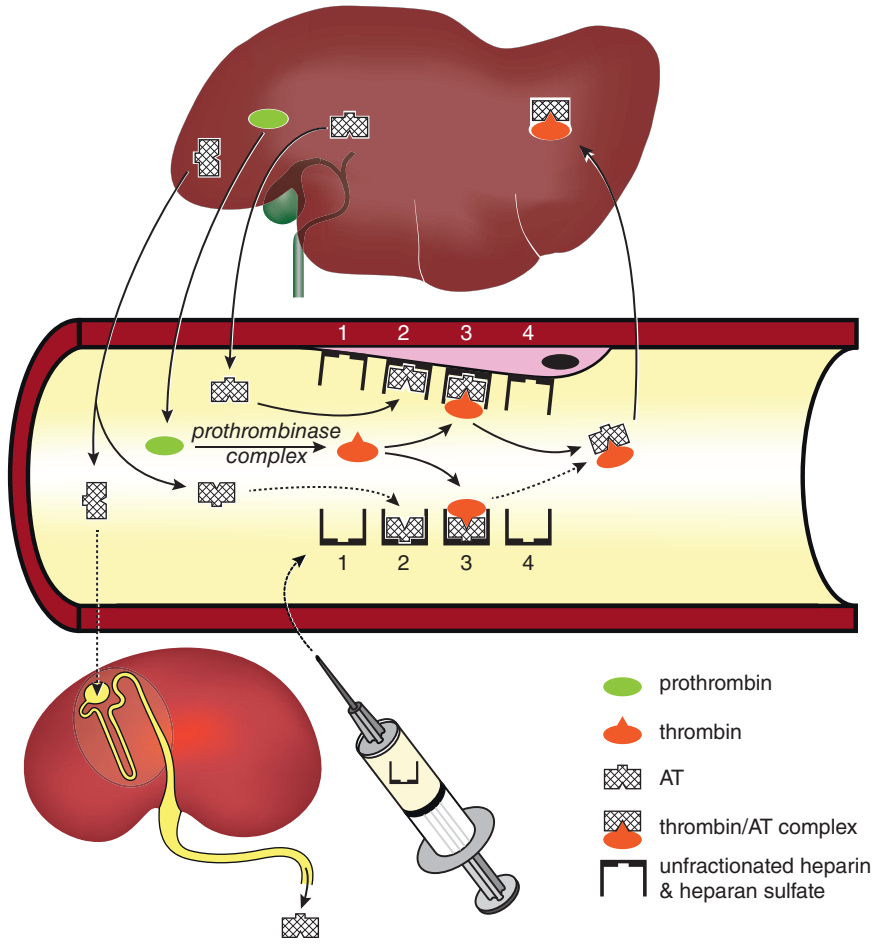


Figure 5.4. Antithrombin pathways. AT is produced primarily by hepatocytes and circulates in the blood, where it can inactivate thrombin (shown here) and all the other coagulation enzymes. As shown schematically, AT activity is markedly enhanced in the presence of circulating unfractionated heparin or heparan sulfate (1). Heparin or heparan sulfate bind to AT (2), inducing a conformational change in AT (note the larger triangular notches) that increases its affinity for thrombin. Thrombin, generated from the activation of prothrombin, then binds to the heparin-AT complex, forming a covalent 1:1 complex with AT while binding to heparin (3). LMWH molecules are too short to bind thrombin and cannot inactivate thrombin, but they induce conformational changes in AT that enable it to bind to and inhibit FXa. The thrombin–AT complexes (TAT) dissociate from the heparin or heparan sulfate and are cleared from circulation by hepatocytes. The heparin and heparan sulfate act as catalysts and are therefore available (4) for forming more complexes. Anticoagulation by AT and heparin limits excessive clotting, but AT and heparin do not inhibit coagulation enzymes bound to fibrin or platelets. Therefore, localized and controlled coagulation can proceed where needed.

- Decreased plasma AT activity and concentration occur via consumption when intravascular coagulation is increased or after injection of exogenous heparin. Decreased concentrations may also occur from decreased hepatic production or from excessive loss due to protein-losing nephropathy or enteropathy. Decreased AT produces a prothrombotic state that heparin cannot counter well because heparin requires AT for most of its function.
- Increased plasma AT activity and concentration may occur with increased hepatic production of AT. This may occur secondary to the production of inflammatory cytokines in cats.

Note: Nonphysiologic pathways are represented by *dotted arrows*.

4. Other circulating inhibitors of coagulation include protein Z–dependent protease inhibitor and its vitamin K–dependent cofactor protein Z, heparin cofactor II, α_2-macroglobulin, and α_1-proteinase. These do not currently play significant roles in clinical diagnostics.

II. Analytical concepts

A. Analytical methods with limited known clinical utility or availability are not discussed in this chapter, though their value may be proven with time. For assays that are discussed, it is important to recognize that assay optimization for one species (e.g., humans) does not necessarily optimize for other species with regard to reagents (e.g., thromboplastins), incubation times, or sample dilutions.[97–102] In addition, assays for routine coagulation testing are not standardized, and many are not optimized for the species being tested. This compromises their sensitivity in detecting impaired coagulation.

B. It is also critical to appreciate that preanalytical processes related to sampling and sample handling can have substantial impact on the accuracy of hemostasis assay results. Therefore, guidelines should be followed whenever possible. Small patient size and uncooperative patients may be unavoidable reasons for suboptimal samples, and efforts to preserve certain veins for other uses may also contribute.

C. Although several studies have assessed the effects of various blood collection and processing methods on hemostasis test results in domestic mammals, applicability of the findings is limited. Lack of an effect in healthy animals may not translate to lack of an effect in diseased or anticoagulated patients. Lack of an effect with certain types of assays may not apply to other types of assays or modifications of the same type of assay. Lack of an apparent effect in one step of the process may be because the effect was masked by problems with a different step, such as delayed exposure of blood to anticoagulant or a suboptimal centrifugation method.

D. Sample collection:

1. The goal is atraumatic collection (one smooth stick directly into the vessel lumen) of free-flowing blood and immediate citrate anticoagulation. Ideally, for most coagulation tests, this may be achieved by clean jugular venipuncture and drawing samples into a citrate vacuum tube.
   a. Drawing into a needle and syringe without anticoagulant and then transferring the sample to a citrate tube is common but not recommended because coagulation (and platelet activation) may begin during venipuncture and progress far enough to alter hemostasis test results prior to mixing with the citrate. This is particularly true of slow draws from smaller veins (e.g., cephalic). If blood is transferred from syringe to tube, it should not be forcefully ejected.
   b. Traumatic venipuncture such as probing with the needle, sampling through a hematoma, applying excessive suction, or exiting and reentering the vein exposes the blood to TF, thus initiating coagulation that can produce clotted samples or cause platelet activation and clumping via generation of thrombin. This commonly shortens clotting times. If collection is difficult, a new vein should be selected. With a mildly traumatic venipuncture, either the first few drops or a complete tube of blood may be discarded to decrease TF in the test sample, but this is not necessary for a "clean stick."
2. Catheters: For routine testing, sampling through catheters may be done after flushing the catheter with 5–10 mL saline and discarding the first blood removed (e.g., 5 mL or 6 times the catheter dead space). Such an approach produced results for *healthy* dogs that did not differ significantly from direct venipuncture results.[103]

3. Immediately after collection, the blood and citrate anticoagulant should be thoroughly mixed by three to four gentle and complete (over and back) inversions of the tube. The sample should not be used if any clots are present.

E. Sample
1. Type: Most coagulation tests require citrated plasma samples. POC instruments may use citrated whole blood or citrated plasma, and certain tests (e.g., ACT, serum FDPs) require a special tube. Seldom used today, the modified Lee–White whole blood clotting time is measured immediately in nonanticoagulated whole blood. EDTA has been evaluated for some tests but would require different RIs and is generally not accepted by laboratories.
2. Tubes: Citrated sample tubes may be made of plastic or siliconized (internally coated) glass. Although statistically significant differences in test results were documented in comparisons of plastic and glass tubes with human samples, differences have been small and rarely of clinical significance.[104]
3. Citrate percentage: Plasma or whole blood should be anticoagulated with trisodium citrate at a 1:9 (anticoagulant to blood) ratio by using 3.2 % or 3.8 % citrate tubes (check with the laboratory for a preference). The citrate percentage has been reported to significantly affect results of some coagulation tests on human samples,[105–108] and it can have statistically significant effects on some results with canine samples.[109,110] However, the differences are usually not of clinical significance, at least for well collected and processed samples from healthy individuals. Because the magnitude of the effect is not known for most conditions, most species, and most assay systems, a standard citrate concentration with RIs generated by using that citrate concentration, instrument, and method is recommended.
4. Citrate to blood ratio: For samples without an increased Hct, it is important to stay within 10 % of the volume needed for a 1:9 citrate to blood volume ratio. Overcitrated samples (too little blood) can have reduced coagulation activity (prolonged times) because the excess citrate in a "short" sample binds more of the Ca^{2+} used in the assay reagent mixture to recalcify the sample, and thus less Ca^{2+} is available to support coagulation. Undercitrated samples (too much blood) might be hypercoagulable (reduced times). Problems associated with suboptimal filling of human samples appear to be greater with 3.8 % citrate tubes than with 3.2 % citrate tubes.[111]
5. Effect of Hct: Hct affects the appropriate amount of citrate to use.[112] With erythrocytosis, plasma will be overcitrated if the routine volume of blood is drawn into a citrate tube because there is less plasma with the same amount of citrate. This may prolong coagulation times because the excess citrate binds too much of the Ca^{2+} added to the assay system.
   a. Decreased citrate to blood ratios are recommended for human samples with Hct > 55 %.[113] A formula (Eq. 5.1) may be used to calculate the citrate volume required for anticoagulating a volume of blood with a given Hct > 55 %:

$$C = 0.00185 \times (100 - Hct) \times V \quad (\mathbf{5.1})$$

C = citrate volume (mL) required for the patient's sample

Hct = patient's blood Hct (%)

V = volume of blood (mL) required for the tube being used

Example : Patient Hct = 70 %; 3 mL citrate tube (contains 0.3 mL fluid)
$C = 0.00185 \times (100 - 70) \times 2.7 = 0.15$ mL
Therefore, remove 0.3 mL – 0.15 mL = 0.15 mL from the tube prior to use

b. The calculated volume of anticoagulant fluid can be used by removing the necessary volume of anticoagulant fluid from a citrate tube through the rubber stopper with a tuberculin syringe (this should maintain the tube vacuum) or with a sterile pipette after uncapping the citrate tube, in which case blood must be collected by syringe and transferred without undue force to the tube, filling to the tube's intended collection volume.

c. In human medicine, undercitration of samples from anemic patients does not cause a clinically significant shortening of PT or PTT, so citrate volumes do not need to be adjusted.[114]

F. Sample processing and stability

1. Time and temperature before processing: Broadly applicable information for sick domestic mammals is limited, but recommendations for human tests are to avoid refrigeration of whole blood because it activates platelets and increases FVII activity, potentially shortening PT.[115,116] It is recommended that samples remain capped and be held at room temperature (18–25 °C) until processing, ideally within 4 h of sample collection but up to 24 h for most tests, including PT. With samples from patients who have not received heparin, PTT prolongations may begin to occur with 12–24 h of storage, whereas samples from heparinized human patients should be harvested within 1 h to avoid neutralization of heparin by platelet release of platelet factor 4. However, platelet factor 4 was not detected in the canine platelet secretome.[1] Storage of canine whole blood at room temperature for 24 h had little effect on plasma PT, but PTT was shortened in many samples and was rarely prolonged compared to promptly tested samples.[117]
2. Centrifugation and plasma harvest
   a. After confirming the absence of clots in the sample, blood should be centrifuged for 10–15 min at a force sufficient to remove most platelets (e.g., 15 min at 1500 × g), but not so great as to cause platelet activation. Because such upper limits are not clearly defined, it is prudent to refrain from higher forces than necessary. The platelet-poor plasma should be removed by plastic pipette. Excessive platelets ($> 10 \times 10^3/\mu L$) remaining in the plasma because of inadequate centrifugation forces will not interfere with PT and PTT assays for routine diagnostic work but will interfere with some tests, e.g., heparin monitoring of human samples via PTT, and tests for other inhibitors of coagulation.[115,118,119] Double centrifugation is needed for some special tests of hemostasis.
   b. Plasma that cannot be tested within 4 h (8 h for routine veterinary screening tests) should be frozen in an additive-free tube. Plasma samples should be rejected if they contain large clots or smaller opaque or gelatinous globules or strands of fibrin.
3. Plasma storage time and temperature: Based on the cumulative information published for domestic mammals, routine screening tests are probably not clinically significantly affected by storage of promptly harvested plasma for up to 8 h at room temperature or 4 °C. Differences in reported findings likely relate to variations in the populations assessed (health versus sick or treated) and in the specific sample collection, processing, and testing methods used. Changes in test results with some long-term stability studies are difficult to interpret because of omitted information about assay reproducibility over the same period.
   a. Room temperature plasma storage: Freshly harvested plasma from *healthy* dogs yielded stable hemostasis results (PT, PTT, TT, AT, specific coagulation factors, and D-dimers) for 48 h when stored at 24 °C, except for mild decreases in clottable [Fbg] at 48 h (it appeared to start dropping by 24 h).[120] However, with room

temperature storage of human plasma, activities of TT, PTT, and FVIII were decreased by 8 h in samples from *heparinized* patients.[121] Mild increases in PTT (but not PT) were measured in thawed, previously-frozen canine plasma samples stored at room temperature for 48 h (but not 24 h).[122]

b. Refrigerated plasma storage: Plasma from *healthy* dogs had less stability at 4 °C than at room temperature.[120] Factor VIII and IX activity were decreased by 48 h (they appeared to be dropping by 24 h), and PTT was prolonged by 72 h (it appeared to be rising by 48 h). In a storage study of citrated canine plasma harvested from donor units, PTT (but not PT) was prolonged after ~1 d, along with mild decreases in FVII activity and clottable [Fbg].[123] PTT, PT, and clottable [Fbg] were stable for 6 h (longest time tested) in plasma from *healthy* horses.[124] Human plasma PT was stable for only 24 h at 6 °C, and FVIII activity was decreasing by 8 h.[121] Samples from *heparinized* patients were stable for 8 h, although activities of cofactors V and VIII were dropping by 8 h.

c. Frozen samples: Human plasma may be stored for 3 mo at −24 °C or for 18 mo at −74 °C before testing, but the use of frost-free freezers should be avoided.[125] Plasma from *healthy* dogs stored at −70 °C had stable results for PT, TT, and clottable [Fbg], but AT activity appeared to be lower and PTT appeared to be more prolonged than in fresh samples.[126] If frozen plasma contains too many platelets, thawing or repeated freezing and thawing can affect some hemostasis assays because procoagulant platelet factors can be released from damaged platelets.

d. When mailing samples for testing, frozen plasma should be packed in ice and mailed to arrive frozen within 24 h. It is up to the sender to assure that the correct plasma to citrate ratio was used, because the lab will not be able to identify if a citrate tube was inappropriately filled once the plasma has been withdrawn. Samples should be tested promptly after thawing rapidly at 37 °C to minimize cryoprecipitate formation and therefore loss of hemostatic factors. Concurrently collecting, processing, and mailing a sample from a healthy patient of the same species can be used as evidence that sample handling did not induce abnormal results.

4. Hemolysis: Samples hemolyzed by in vitro factors should not be tested because coagulation and platelets may have been activated by the same factors responsible for hemolysis. This is most likely to shorten coagulation times.

G. Instruments: A variety of automated coagulation analyzers, including POC instruments, have largely replaced manual and fibrometer methods of measuring coagulation times in chronometric (time-based) assays for human and veterinary samples. Specific methods and sample requirements vary with the analyzer. Endpoint detection of a fibrin clot varies with the type of analyzer, and some incorporate multiple detection systems.

1. Electromechanical methods

a. Electrical conductance endpoints: Increased electrical conductance is detected between a stationary electrode and a moving electrode when fibrin forms and maintains a connection (soft clot) between them (fibrometers).

b. Electromagnetic endpoints: With one method, a magnetic sensor detects electromagnetically-induced oscillation of a metal ball within the sample. As the clot forms and the sample becomes more viscous, reduction in the movement of the ball to a predetermined level signals the assay endpoint. With another method, sample cuvettes contain a metal ball that is rotated magnetically until the ball is incorporated into the fibrin clot, thus causing a decrease in current that marks the endpoint of the assay.

c. Electrochemical (amperometric) endpoints: Coagulation generates thrombin, which cleaves a peptide substrate to produce an electrochemical signal that is converted to typical coagulation units based on an algorithm.

2. Photo-optical methods
   a. Turibidometric and nephelometric methods detect changes in light transmission or light scatter, respectively, as fibrin forms, and some provide additional information via clot curves that show changes over time. Endpoints are based on predetermined levels of absorbance or light scatter compared to baseline, and the wavelengths of light that are assessed minimize interference by lipemia or icterus.
   b. Optical sensors are used to detect blood flow in the cartridges of some POC analyzers (Coag Dx, SCA2000, and VETSCAN VSpro Coagulation Analyzers). Coagulation endpoints are defined by a decrease in flow rate to a predetermined value.
3. VET (see Global Assessment of Hemostasis)

H. General analytical approach: In vitro analysis enables independent testing of different branches of the coagulation web (simplified to a "Y") so that defects may be localized within the surface-induced (intrinsic), TF (extrinsic), or common pathways (Figure 5.5). Testing generally begins with common screening tests, usually paired PT and PTT. More-specialized tests are used when indicated.

I. Severe hypothermia may contribute to hemorrhage, in part because coagulation is a temperature-sensitive enzymatic pathway. However, coagulation testing is routinely done at 37 °C (near body temperature), so routine assays may not identify hypothermia-induced coagulation abnormalities.

III. Activated partial thromboplastin time (APTT, aPTT, or PTT): a screening coagulation test of the surface-induced (intrinsic) and common pathways

A. Principle and method

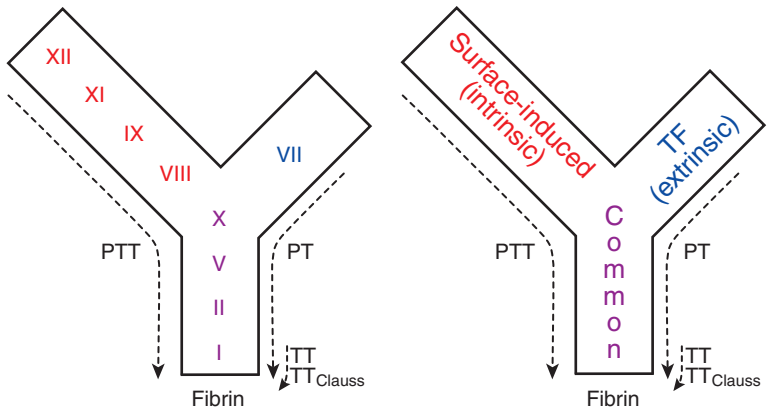


Figure 5.5. Schematic representation of the coagulation cascade as evaluated by in vitro screening coagulation tests. Fibrin formation is the end point for each test. PTT (and ACT) evaluate coagulation factors in the surface-induced (intrinsic) pathway (factors XII, XI, IX, and VIII) and in the common pathway (factors X, V, II, and I). PT evaluates the short TF pathway (FVII) and the common pathway. TT and TT_{Clauss} assess only the conversion of Fbg (factor I) to fibrin with the addition of thrombin. TF and FXIII are not assessed.

1. Surface-induced (contact) activation: Citrated test plasma is incubated (37 °C) with an excess of procoagulant phospholipids (partial thromboplastin) and a surface activator, e.g., silicates, including kaolin (hydrated aluminum silicate), or ellagic acid. This activates the contact factors, but chelation of fCa^{2+} by citrate in the sample limits activation beyond FXIa.
   a. Partial thromboplastin reagents consist of procoagulant phospholipids devoid of TF (hence "partial"), and therefore the reagent is unable to activate the TF pathway via FVII.
   b. In the original partial thromboplastin assay, the glass tube (rather than added particulates) was the surface activator, and the clotting time was slow and less reproducible.[127] To differentiate that test from the subsequent test with added particulates, the later test was referred to as the *activated partial thromboplastin time* (APTT or aPTT). However, contact factors were activated with each method and the original method is obsolete, so many people now refer to the test simply as the PTT, as is done in this text.
2. After a defined incubation time, a measured amount of prewarmed (37 °C) calcium chloride is added to counteract the effects of citrate and enable the cascade of coagulation to proceed to the formation of fibrin monomers, which polymerize to form an insoluble fibrin clot that is detected optically or electromechanically, depending on the instrument. *The time from addition of calcium chloride to clot detection is the PTT.*
3. Units: seconds (usually reported to nearest 0.1 s)

B. Interpretive considerations
1. Laboratory-specific and species-specific RIs must be used because results may vary substantially with variations in species, analyzers, reagents, and protocols.[128] Values greater than a valid upper reference limit should be considered prolonged. As with other RIs based on the central 95 % of healthy individuals, minor prolongations may or may not be clinically significant. Other laboratory and clinical information should be considered.
   a. Without RIs, results were often interpreted by comparison to values from a concurrently tested healthy animal of the same species. This is not recommended because a single comparison animal is not representative.
   b. Some people have considered an increase to be significant when the result is at least 20 % or 25 % over the upper reference limit or 1.5–2.0× the reference mean. However, smaller increases may be clinically significant, as the sensitivity of many PTT assays to factor deficiencies is relatively low (i.e., a large decrease in factor activity is required for a mild prolongation of the PTT).
2. Although it varies with the specific assay, PTT results are typically prolonged when there is about a 70 % decrease in the activity of a single coagulation factor (30 % of expected activity). Milder reductions in individual factor activities can be detected when multiple factors are affected.
3. As equine data indicate, values in healthy newborns (< 24 h old) may be greater than RIs established for adults.[129,130]
4. PTT assayed at 37 °C may overestimate in vivo coagulation in markedly hypothermic patients because enzyme activities are temperature dependent.[131,132]
5. Excessive citrate, high Hct, or improper handling (delayed processing, an old sample, or an inappropriate temperature) may prolong PTT. Preanalytical activation of coagulation may reduce the PTT by premature activation of coagulation factors.

C. A prolonged PTT indicates decreased in vitro function of the surface-induced or common pathways by one or more of the following causes (Table 5.6) that are differentiated by other hemostasis tests, other laboratory tests, and clinical findings.
   1. Hereditary factor deficiencies
      a. Carriers of hemophilia cannot be detected by PTT because they have only a moderate decrease in factor activity (approximately 50 % of normal).
      b. Pure deficiencies of either FXII or PK are not associated with clinical hemorrhage, but they are associated with prolonged PTT results in most assay systems.[100] FXII deficiency is relatively common in cats, occurring in multiple breeds.[133] PK deficiency is rare. A prolonged PTT caused by PK deficiency may be corrected

Table 5.6. Disorders or conditions that may cause a prolonged PTT

Deficiencies of functional surface-induced or common-pathway coagulation factors
Acquired (usually multiple defects)
Decreased production
Hepatic disease (necrosis, cirrhosis, hepatitis, lipidosis, neoplasia, shunts)*
Vitamin K antagonism or deficiency
Decreased vitamin K recycling in hepatocytes: ingestion of anticoagulant rodenticide,* excess coumadin or phenoprocoumon, moldy sweet clover (*Melilotus* spp.); defective hepatocytes in hepatocellular carcinoma
Decreased vitamin K absorption: biliary obstruction,* infiltrative bowel disease, chronic oral antimicrobials, exocrine pancreatic insufficiency
Increased coagulation factor inactivation and consumption
Disseminated intravascular coagulation or coagulopathy*
Localized consumptive coagulation or coagulopathy
Venom-induced consumptive coagulopathy
Increased endogenous anticoagulant activity and/or fibrin(ogen)olysis
Acute traumatic coagulopathy
Certain anticoagulant envenomations
Dilution of coagulation factors: acute, massive blood loss treated with massive transfusion of plasma-poor fluid, e.g., packed RBCs and crystalloid or colloid fluids, including Oxyglobin solution
Hereditary (usually single defect, but may be multiple)
Surface-induced (intrinsic) pathway: PK, FXII, FXI, FIX, FVIII
Common pathway: FX, FV, FII, Fbg (FI)
Inhibition of surface-induced or common-pathway coagulation factors: exogenous or endogenous heparin,* direct-acting oral anticoagulants (anti-IIa or Xa), FDPs (fibrinolysis),* antiphospholipid antibodies, antibodies to coagulation factors
Unknown or multiple mechanisms
Anaphylaxis
Zinc toxicosis

* A relatively common disorder, condition, or mechanism

Note: ACT may also be prolonged in most of these conditions, but ACT prolongation requires greater deficits in factor activities.

by increasing the incubation time of plasma with the particulate surface activator or by using ellagic acid for surface activation.[134]

2. Acquired factor deficiencies from decreased production, defective production, or increased consumption
3. Increased endogenous anticoagulation and fibrin(ogen)olysis including heparin and tryptase release from mast cells with severe anaphylaxis or mast cell neoplasms[135,136]
4. Massive hemodilution
5. Coagulation inhibitors including FDPs that form with fibrinolysis and may be accompanied by hypofibrinogenemia and, in the case of thrombolytic therapy, heparin; other inhibitors are direct-acting oral anticoagulants, potentially pentosan polysulfate (a heparinoid), and rare interfering antiphospholipid or anti-factor antibodies

D. A shortened PTT is not reliable for detecting hypercoagulability. It may occur because of in vitro activation of coagulation associated with suboptimal venipuncture or sample processing. If it is repeatable in the absence of such preanalytical errors, it may reflect the bottom 2.5 % of healthy patients with values below a routinely determined lower reference limit, or it may reflect a procoagulant state. In dogs, decreases in PT or PTT were associated retrospectively with an increased incidence of thrombosis, suspected thromboembolism, and increased [D-dimer].[137] In people, decreases are reported to occur with increases in Fbg, FV, or FVIII as may be seen in inflammation.[138]

E. PTT has been used to monitor unfractionated heparin therapy, but targets of prolongation used for people (e.g., 1.5–2.5 × baseline) do not appear to reflect appropriate anticoagulation in domestic mammals. PTT was less prolonged in dogs than in people for a given plasma heparin activity, possibly because of greater FV and FVIII activity in dogs.[139] Target prolongations vary with the PTT assay and reagents, in part because partial thromboplastin reagents have different sensitivities to heparin.[140,141] It has been recommended that any monitoring of unfractionated heparin with PTT should be based on site-specific comparisons of PTT and anti-FXa activity.[142]

F. LMWH has minimal effect on PTT because of reduced anti-FIIa activity;[143] anti-FXa assays can be used to monitor LMWH therapy (see Anti-FXa (anti-Xa) activity).

IV. Prothrombin time (PT), also called one-stage prothrombin time (OSPT): a screening coagulation test of the TF and common pathways

A. Principle and method

1. Test plasma and a Ca^{2+}-thromboplastin reagent containing phospholipid and excess TF (so-called complete thromboplastin) are separately prewarmed (37 °C).
2. A measured amount of the prewarmed Ca^{2+}-thromboplastin reagent is forcibly added to a measured amount of the prewarmed plasma so that TF activates FVII in the TF pathway. Activation should proceed along the common pathway to form fibrin monomers, which polymerize to form an insoluble fibrin clot that can be detected optically or electromechanically. *The time from plasma and thromboplastin mixing to clot detection is the PT.*
3. A heparin inactivator is generally included in PT assays.
4. Different thromboplastin reagents produce different results.[144,145] Some appear to be affected by PIVKA, which interfere with the generation of thrombin and inhibit coagulation in vitro.
5. In modified or optimized PT methods, reagents and plasma can be diluted to prolong PT and increase the analytical sensitivity for detecting abnormalities.[146,147] Fbg may be

added to diluted samples to ensure adequate Fbg concentration, in which case patient hypofibrinogenemia would not be detected by a prolonged PT.

6. POC instruments are available, and manufacturer recommendations should be followed.
7. Units
   a. Seconds (usually reported to nearest 0.1 s)
   b. PT may be reported as a unitless INR for monitoring warfarin therapy, particularly in human laboratories, but such values have limited value (see next Section F).

B. Interpretive considerations are the same as previously described for PTT. In one study of various PT reagents and methods, the FVII activity necessary to keep PT WRI varied from 16 % to 39 %.[102] Unless used to monitor warfarin therapy, PT values are best interpreted relative to a valid RI.

C. Prolonged PT indicates decreased in vitro function of the TF or common pathways (Table 5.7). PT is typically not sensitive to heparin because of the presence of heparin inactivators in most thromboplastin reagents, but it may be prolonged with certain thromboplastin reagents when blood contains therapeutic heparin concentrations.[148]

D. Shortened PT is not reliable for detecting hypercoagulability. It may occur with activation of coagulation during venipuncture, but with many PT assays, shortening is limited by already relatively short clotting times. If it is repeatable in the absence of preanalytical errors, it may reflect the bottom 2.5 % of healthy patients with values below a routinely determined lower reference limit, or it may reflect a procoagulant state. In dogs, decreases in PT or PTT were retrospectively associated with an increased incidence of thrombosis, suspected thromboembolism, and increased [D-dimer].[137] In people, decreases are reported to occur with increased [Fbg] or FV activity.[138]

E. PT is used to monitor warfarin therapy (target PT $\cong$ 1.5–2.0 × baseline).

F. *INR*, a unitless ratio (Eq. 5.2) developed for monitoring warfarin therapy in people, is used widely in human medicine to help standardize the reporting of PT values by correcting for differences in instrumentation and thromboplastin reagents among laboratories.[149] In Eq. 5.2, the *ISI* (International Sensitivity Index) is a number each thromboplastin manufacturer determines by testing each lot of thromboplastin reagent with a particular PT method against an international reference thromboplastin preparation. (More specifically, the ISI is 1/slope of the regression line graphing the log PT for the test thromboplastin

Table 5.7. Disorders or conditions that may cause a prolonged PT

Deficiencies of functional coagulation factors in the TF or common pathway
Acquired (usually multiple defects); see Table 5.5*
Hereditary (usually single defect)
TF pathway: FVII
Common pathway: FX, FV, FII, Fbg (FI)
Inhibition of coagulation factors in the TF or common pathway: direct-acting oral anticoagulants (anti-IIa or Xa), FDPs,* antiphospholipid antibodies, antibodies to coagulation factors, rarely heparin
Unknown or multiple mechanisms
Anaphylaxis
Zinc toxicosis

* A relatively common disorder, condition, or mechanism

against the log PT of the reference thromboplastin using a particular assay and samples from 20 healthy people and 60 people stabilized on chronic warfarin anticoagulation.)[150] The ISI reflects the relative sensitivity of the reagent to factor deficiencies based on the relationship between PT values for healthy people and people receiving warfarin. Thromboplastins with higher ISI values are less sensitive than thromboplastins with lower ISI values.

$$\text{INR} = \left(\frac{\text{Patient PT}}{\text{Reference PT}}\right)^{\text{ISI}} \qquad (\mathbf{5.2})$$

1. Although the INR enables more meaningful comparisons of human PT results among laboratories, it does not eliminate significant interlaboratory differences.[150]
2. INR is not applicable to domestic mammals because appropriate species-specific ISI values are not available and would generally be cost-prohibitive to generate.[147] Also, warfarin monitoring is not the major reason for PT testing in domestic mammals, and the INR is not as applicable to other causes of prolonged PT that involve different patterns of factor deficiency.
3. Although not appropriate for domestic mammals in the first place, if a human laboratory reports an INR for a veterinary species, it is likely that the reference PT in the INR calculation is the human value and not an appropriate species-specific value. Using an inappropriate reference mean or single control sample can generate a markedly different INR and interpretation.
4. Reporting the PT as a ratio of the patient result to the reference mean improves comparison among different assays as compared to comparing PT in seconds, but such comparisons are still problematic.
5. Although INR calculations are not directly applicable to domestic species, they reflect the fact that not all thromboplastin reagents and assays yield the same PT results and that results cannot be compared directly. The following examples used in Eq. 5.3 illustrate this concept. The reference PT is the mean of the RI.
   a. Different patients, different thromboplastin reagents, but same PT value
      (1) Patient 1, ISI = 1.5: PT = 12.0 s; reference mean = 8.0 s (Eq. 5.3a)
      (2) Patient 2, ISI = 2.3: PT = 12.0 s; reference mean = 8.0 s (Eq. 5.3b)
      (3) Patients 1 and 2 had the same PT (12.0 s) when patient 2 was tested with a less sensitive thromboplastin reagent (which had a greater ISI). The amount of anticoagulation was actually greater for patient 2, despite the same PT value. This is reflected by a greater INR.
   b. Different thromboplastin reagents but the same patient sample
      (1) Patient 1, ISI = 1.5: PT = 12.0 s; reference mean = 8.0 s (Eq. 5.3a)
      (2) Patient 1, ISI = 2.3: PT = 10.3 s; reference mean = 8.0 s (Eq. 5.3c)
      (3) When patient 1 was tested with the less sensitive reagent (which had a greater ISI), the PT was only 10.3 s, but the INR, and therefore degree of anticoagulation, was unchanged.

$$\text{Patient\#1 INR} = \left(\frac{12.0}{8.0}\right)^{1.5} = 1.8 \qquad (\mathbf{5.3a})$$

$$\text{Patient\#2 INR} = \left(\frac{12.0}{8.0}\right)^{2.3} = 2.5 \quad (\mathbf{5.3b})$$

$$\text{Patient\#3 INR} = \left(\frac{10.3}{8.0}\right)^{2.3} = 1.8 \quad (\mathbf{5.3c})$$

V. Proteins induced by vitamin K antagonism or absence (PIVKA): PIVKA are the non- or incompletely γ-carboxylated vitamin K–dependent coagulation factors (e.g., des-γ-carboxy prothrombin, often referred to as PIVKA-II because prothrombin is FII) that are produced by hepatocytes during periods of vitamin K antagonism or deficiency (see Major Bleeding Disorders, Section II.B).[82] In these conditions, plasma concentrations of normally carboxylated coagulation factors are decreased, and PIVKA are secreted and circulate in the blood at increased concentrations. The potential of PIVKA to become activated to functional coagulation enzymes is severely limited.
   A. Human PIVKA assays: Clinical immunoassays are used in human medicine to specifically and directly measure plasma [PIVKA], but they are not used in veterinary medicine. They can detect abnormalities in the vitamin K–dependent factors that PIVKA-sensitive coagulation tests cannot.[151] Charge differences between PIVKA and their carboxylated forms also enable detection by high-performance liquid chromatography.[152] Increased plasma [PIVKA] may occur with vitamin K deficiency, vitamin K antagonism, or as a result of production by malignant hepatocytes as documented in some human patients with hepatocellular carcinomas.[153]
   B. The Thrombotest PT, a modification of the PT assay that is PIVKA sensitive, has been referred to as a PIVKA test, but it is not specific for PIVKA and does not directly measure [PIVKA].[154,155] It is a PT test that detects decreased activities of coagulation factors II, VII, and X from any cause. FV and Fbg are not assessed because they are provided in the assay.
      1. If PIVKA are present in the sample, the Thrombotest PT will be further prolonged because PIVKA appear to act as competitive substrates that delay the generation of thrombin. However, PIVKA do not appear to interfere with functional factors in vivo.[156]
      2. Causes of a prolonged Thrombotest PT
         a. Vitamin K antagonism or deficiency: Compared to the most commonly used PT assays, the Thrombotest PT may have greater analytical sensitivity to detect mild vitamin K antagonism or deficiency, and it may help differentiate these conditions from other coagulopathies when appropriately high decision thresholds are used. However, when the investigators' recommended decision thresholds were used, diagnostic sensitivity and specificity for vitamin K antagonism or deficiency were similar for the Thrombotest PT (78.6 % and 99.7 %, respectively) and a traditional PT (83.3 % and 97.2 %, respectively).[157,158]
         b. Other acquired and hereditary coagulopathies as for PT, excluding FV and Fbg deficiencies (Table 5.7)[144,159,160]
         c. Cats with a variety of hepatic, biliary, or inflammatory bowel diseases had prolonged Thrombotest PT values, often without clinical evidence of a bleeding tendency.[154] In a subset of these cats treated with vitamin K_1, Thrombotest PT values were WRI 3–5 d after treatment was instituted, indicating that prolonged Thrombotest PT values were at least transiently responsive to vitamin K. Although the assay appears to detect relatively mild deficiencies in cats, its diagnostic specificity for clinically significant bleeding tendencies is unknown.
         d. Endogenous or exogenous heparin[157]

VI. Thrombin time (TT), also called thrombin clotting time (TCT): This test assesses the thrombin-induced conversion of Fbg to an insoluble fibrin clot without the need for the patient sample to generate the thrombin. It helps differentiate terminal common-pathway problems – hypofibrinogenemia, dysfibrinogenemia, and effects of heparin, FDPs, and paraproteins – from other causes of prolonged PT and PTT.

A. Principle and method

1. Thrombin is added to prewarmed test plasma (37 °C). Thrombin cleaves Fbg to form monomers, which polymerize into an insoluble fibrin clot that can be detected optically or electromechanically, depending on the instrument. *The time from thrombin addition to detection of a fibrin clot is the TT.*
2. Units: seconds (usually reported to nearest 0.1 s)

B. Interpretive considerations: TT should be differentiated from the TT_{Clauss} (Fbg) assay (see next Section VII), which uses diluted plasma and high thrombin concentrations to better measure clottable [Fbg] with less interference by heparin and FDPs. Lab-specific RIs should be used for interpretation because of method variations.[139]

C. Prolonged TT

1. Hypofibrinogenemia or afibrinogenemia (see Table 5.8 and next Section VII)
2. Dysfibrinogenemia (see Table 5.8 and next Section VII)
3. Heparinized patient or sample: Unfractionated heparin interferes with thrombin activity via AT, so TT can be used to monitor unfractionated heparinization.[139] TT prolongation by heparin can be normalized with heparin neutralization. TT is affected relatively little by LMWH.[143]

Table 5.8. Disorders or conditions that may cause decreased clottable [Fbg] (Fbg_{Clauss}, TT_{Clauss}) or TT (TCT)

Deficiencies of functional Fbg
Acquired
Decreased production: hepatic disease, portosystemic shunts, microvascular dysplasia
Abnormal production (rare): hepatic disease, hepatic or nonhepatic neoplasms
Increased consumption
Disseminated intravascular coagulation or coagulopathy*
Localized consumptive coagulation or coagulopathy
Acute traumatic coagulopathy
Certain envenomations (thrombin-like or profibrinolytic factors)
Fibrinolytic therapy (t-PA, streptokinase, urokinase)
Possibly *Angiostrongylus vasorum* infections
Possibly delayed hemorrhage in greyhounds
Hemodilution: acute, massive blood loss treated with massive transfusion or plasma-poor fluids, e.g., RBCs and crystalloid or colloid fluids, including Oxyglobin
Hereditary
Afibrinogenemia or hypofibrinogenemia
Dysfibrinogenemia
Interferents: heparin contamination, direct-acting oral thrombin inhibitors, high [FDP], autoantibodies to Fbg

* A relatively common disorder, condition, or mechanism

4. Increased [FDP]: FDPs interfere with fibrin polymerization and with thrombin's action on Fbg.[161] Fibrinolytic therapy can be monitored with the TT.
5. Direct-acting oral anti-IIa anticoagulants (e.g., dabigatran)
6. Paraproteinemia: Abnormal immunoglobulins from plasma cell myelomas can interfere with fibrin polymerization.[162]
7. Systemic amyloidosis (reported in people): Plasma from affected patients may contain an inhibitor of fibrin polymerization.[163]
8. Hyperfibrinogenemia: Hyperfibrinogenemia rarely has been reported to paradoxically prolong the TT in samples with no PT or PTT prolongations and no increase in FDPs.[164,165] Proposed explanations include Fbg interference with thrombin or with fibrin assembly.
9. TT is unaffected by decreased activity of any coagulation factor (including FXIII) other than Fbg.

D. Shortened TT: This may occur with increased or activated clotting factors (e.g., from traumatic collection), or with soluble fibrin in the sample, but it is not a reliable measure of hypercoagulability.

VII. Fibrinogen, clottable (TT_{Clauss}, Fbg_{Clauss}): This measurement of clottable [Fbg] is the plasma concentration of Fbg measured chronometrically as Fbg activity by using the von Clauss modification of the TT assay (TT_{Clauss} or Clauss method).

A. Principle and method
1. As for the TT assay, thrombin added to prewarmed test plasma (37 °C) cleaves Fbg and generates an insoluble fibrin clot that can be detected optically or electromechanically. *The time from thrombin addition to detection of a fibrin clot is the* TT_{Clauss} (in seconds).
2. In contrast to the TT assay, the plasma sample is diluted so that Fbg is rate limiting, and greater concentrations of thrombin are used to largely override inhibitors such as heparin and FDPs. Commercial assays vary in plasma dilution factor, thrombin source, and thrombin activity concentration.
3. Samples with known clottable [Fbg] are used to construct a reference (standard) curve of TT_{Clauss} against [Fbg]. TT_{Clauss} is inversely proportional to [Fbg]. Test plasma clottable [Fbg] is determined from the standard curve.
4. Units: TT_{Clauss} is measured in seconds, but values for clottable [Fbg] are read from the reference (standard) curve and reported in units of mg/dL or µmol/L.
5. Unit conversion: mg/dL × 0.0294 = µmol/L (SI unit)
6. This method is used in a small on-site analyzer (VETSCAN VSpro) for equine [Fbg] in citrated plasma. Values agreed well with other analyzers when WRI; accuracy and precision of hypofibrinogenemic samples were not reported.[166]

B. Interpretive considerations
1. Plasma [Fbg] reflects the balance between production and consumption. Accelerated consumption of Fbg during hypercoagulable states may be masked by increased production associated with inflammation or pregnancy.[86,167]
2. As equine data indicate, values in healthy newborns (< 24 h old) may be lower than RIs established for adults.[129,130]

C. Increased clottable [Fbg] (TT_{Clauss}): increased Fbg production or hemoconcentration (see Chapter 7); hyperfibrinogenemia is common in prothrombotic states, but it does not appear to be an initiator of thrombosis[168]

D. Decreased clottable [Fbg] (TT_{Clauss}) (Table 5.8)

1. Because the assay is functional, decreased clottable [Fbg] by the TT_{Clauss} method may reflect hypofibrinogenemia, dysfibrinogenemia, or, if present in high concentrations, interferents of fibrin clot formation. With dysfibrinogenemia, polymerization of fibrin is impaired because of abnormal Fbg molecules. With hypofibrinogenemia or afibrinogenemia, there is an inadequate amount of Fbg to form a normal fibrin clot in vitro or thrombus in vivo.[21,169–171]
    a. Decreased Fbg production: This can occur when there is a decrease in functional hepatic mass (e.g., cirrhosis). In addition, synthesis of abnormal Fbg (dysfibrinogenemia) may occur with hepatic disease; in people, it appears that this is largely due to a posttranslational increase in sialic acid residues which increase the negative charge of Fbg and impair its polymerization. Paraproteins produced in lymphoid neoplasms can interfere with polymerization, as can autoantibodies to Fbg (not documented in domestic mammals). Rare hepatic or nonhepatic neoplasms may produce dysfunctional Fbg, but this has not been documented in domestic mammals. Hereditary deficiencies or dysfibrinogenemia are rare.
    b. Increased Fbg consumption: This usually occurs in association with consumptive coagulopathies (disseminated, localized, acute traumatic, or venom-induced) in which thrombin cleavage of Fbg occurs faster than Fbg production to replace it. It can also occur with increased plasmin-mediated degradation of Fbg as occurs with therapeutic use of plasminogen activators, or with primary fibrinolytic conditions, which may include some *Angiostrongylus vasorum* infections, delayed postoperative hemorrhage in greyhounds, and certain envenomations. Thrombin- and plasmin-mediated processes may occur together.
    c. Marked hemodilution: Massive infusion of plasma-poor fluids to treat massive, acute blood loss may expand the blood volume enough to cause transient hypofibrinogenemia (see Major Bleeding Disorders, Section II.D).
2. High concentrations of heparin occurring (i) after a bolus injection, (ii) with inappropriate blood collection from a heparinized catheter, or (iii) with blood collected into a heparinized tube may prolong the TT_{Clauss} and falsely decrease the clottable [Fbg] result.
3. Direct-acting oral anti-IIa anticoagulants (e.g., dabigatran): Accidental ingestion or therapeutic use of thrombin inhibitors is expected to prolong TT_{Clauss}.
4. FDPs have a minimal effect on TT_{Clauss} in human samples unless present at high concentrations concurrently with hypofibrinogenemia, in which case they may falsely decrease the estimate of [Fbg]. Similar minimal effect is expected in dogs.

E. Other methods for measurement of decreased [Fbg] (see Chapter 7)
1. [Fbg] may be measured by detection of Fbg antigen in various immunoassays using anti-Fbg antibodies. Prolonged TT and TT_{Clauss} (decreased clottable [Fbg]) with decreased Fbg antigen indicates hypofibrinogenemia. Prolonged TT and TT_{Clauss} without decreased Fbg antigen supports the presence of a dysfibrinogenemia (rare).
2. [Fbg] determined by heat precipitation and depth of the Fbg precipitant layer as a percentage of the plasma layer (Millar's method, see Chapter 7) can probably detect moderate and severe hypofibrinogenemia if appropriate RIs are generated.[172] This method was applied to the QBC VetAutoread for use in horses, but reports of hypofibrinogenemic samples were not found.[173] In contrast, [Fbg] determined by heat precipitation and refractometry (Schalm's method) is not precise or accurate enough to reliably detect hypofibrinogenemia (see Chapter 7 for heat precipitation

methods). Some other automated Fbg precipitation methods may be able to detect hypofibrinogenemia (see Chapter 7).

3. VET results are substantially affected by [Fbg], low concentrations appearing as hypocoagulability and high concentrations appearing as hypercoagulability, but [Fbg] is not specifically assessed.
4. Clottable [Fbg] has been measured nonchronometrically by spectrophotometric assessment of protein absorbance after washing and dissolving a clot in alkaline urea or other solvents (most protein in the clot is Fbg). It has also been done gravimetrically by completely drying and weighing the clot, but these methods are not suitable for routine diagnostics.
5. PT-derived [Fbg] is not recommended but has been widely measured in human medicine. PT is determined for a series of plasma samples with different known concentrations of Fbg, and the generated calibration curve is used to estimate [Fbg]. Because other factors affect the PT, this is not an assessment of only [Fbg]. Substantial bias was reported between PT-derived and TT_{Clauss}-derived [Fbg] in canine samples.[174]

VIII. Other specific coagulation factor assays (II, VII–XII, PK, HK, and XIII): Besides Fbg assays, other specific coagulation factors are usually assessed by clotting assays in specialized veterinary laboratories when screening tests and clinical findings suggest factor deficiencies. The assays may be used to characterize acquired deficiencies but usually are done to identify hereditary factor deficiencies in clinically affected animals (Table 5.9).

A. Clotting assays for specific factors assess the ability of patient plasma to correct the PT or PTT of specific factor-deficient plasma samples. PTT is used for evaluating intrinsic factors (XI, IX, and VIII) and can be used for common-pathway factor deficiencies. PT is used for evaluating FVII or common-pathway factor deficiencies.
   1. Prolonged PTT or PT of the known factor-deficient plasma usually corrects (becomes WRI) with a 1:1 dilution of patient plasma if the patient plasma contains the missing factor (substrate), because that will supply at least 50 % of normal activity, enough to prevent prolongation with most assays. However, the PTT or PT will not correct if patient plasma has the same factor deficiency.
   2. To determine the amount of factor activity in the test plasma, a reference curve of coagulation time versus reference plasma dilution is generated from mixtures of known factor-deficient plasma and serial dilutions of species-specific pooled plasma considered to have 100 % factor activity prior to dilution. By using the reference curve, observed clotting times for dilutions of the test plasma are converted to % activity relative to the reference plasma pool. Alternatively, units of U/dL, U/mL, or U/L (SI unit) may be used, where patient values are assigned a value relative to the plasma pool that is considered to have activity of 100 U/dL, 100 U/mL, or 100 U/L, respectively.

B. Chromogenic assays may also be used to assess certain specific factors. They assess the capability of a specific factor or cofactor to cause enzymatic cleavage of a chromogenic substrate, thus producing a detectable color change proportional to the factor or cofactor activity in the sample.[175] Examples include FII (prothrombin)[176] and FXa assays,[141] the latter being used in the anti-FXa assay to assess the activity of unfractionated heparin or LMWH in a sample.

C. A FVII ELISA can measure canine immunoreactive [FVII], so it can detect FVII deficiency but not hypofunctional FVII due to vitamin K deficiency or antagonism.[177]

Table 5.9. Congenital coagulation factor deficiencies reported in domestic mammals

Pathway Factor	Condition	Species	Expected test results		
			PTT	PT	TT[a]
Tissue factor					
VII	FVII deficiency	Dogs	WRI	↑	WRI
Surface-induced					
PK	PK deficiency	Dogs, horses	↑	WRI	WRI
XII	Hageman trait	Dogs, cats*	↑	WRI	WRI
XI	Hemophilia C	Dogs, cats, cattle	↑	WRI	WRI
IX	Hemophilia B[b]	Dogs,* cats	↑	WRI	WRI
VIII	Hemophilia A[b]	Dogs,* cats, cattle, horses	↑	WRI	WRI
Common					
X	FX deficiency	Dogs, cats	↑	↑	WRI
II	Hypoprothrombinemia	Dogs	↑	↑	WRI
I	Hypo- or afibrinogenemia	Dogs, cats,[c] goats	↑	↑	↑
Combined					
II, VII, IX, X	Vitamin K–dependent multifactor deficiency	Cats (Devon rex), possibly dogs	↑	↑	WRI
Fibrin stabilization					
XIII	FXIII deficiency	A dog	WRI	WRI	WRI

[a] Expected findings apply to TT and TT_{Clauss}. TT_{Clauss} results are usually reported as [Fbg], which would be decreased for factor I (Fbg) deficiency and WRI for all the other deficiencies.
[b] These conditions have an X-linked recessive transmission and rarely occur in females. The other disorders are autosomal and tend to be less severe.
[c] An apparent congenital Fbg disorder with hypofibrinogenemia in Maine Coon cats
* Relatively common coagulopathy compared to others
Note: Hemorrhage is not associated with isolated PK or factor XII deficiency. Concurrent hereditary or congenital factor deficiencies also occur. Unpublished reports are not included.

D. FXIII deficiency does not affect PT or PTT assays because the endpoints of these assays occur early in the clotting process. Evidence for FXIII deficiency, which can be a clinically significant congenital deficiency, may be obtained from VET or clot solubility testing.[178,179]
   1. VET is expected to yield a prolonged CFT or K and a decrease in maximum clot firmness without a prolonged clotting time.
   2. Clot solubility test: After a clot is formed by addition of thrombin/Ca^{2+} to a citrated plasma sample, it is suspended in a solution of 1 % monochloroacetic acid or 5 M urea, and the time required for clot solubilization at 37 °C is assessed over 24 h. With severe FXIIIa deficiency (< 1 % activity, with no recent plasma transfusion), the reduced fibrin cross-linking shortens the clot solubility time. A sample from a healthy individual should be tested concurrently as a control. Hypofibrinogenemia and dysfibrinogenemia should be excluded first because they may also shorten the clot solubility time.

IX. Dilute Russell's viper venom time (dRVVT): This test is not widely offered or commonly used in veterinary medicine. Venom of the Russell's viper (*Daboia russelii*) contains enzymes that activate factors X and V, allowing functional assessment of the common pathway (factors X, V, II, and I). Modification of the original RVVT by using the venom and phospholipid at low, rate-limiting concentrations yielded the dRVVT, which is the most-used form of the test. A cationic heparin neutralizer is often included so that therapeutic concentrations of unfractionated heparin do not interfere.

A. As a low-phospholipid test (if test plasma is appropriately harvested after adequate centrifugation to minimize platelets), it is relatively sensitive to antiphospholipid antibodies (lupus anticoagulants). Detecting such antibodies is its major use.

B. Prolonged dRVVT (considered a positive screen) can be compared to results after (i) a 1:1 dilution with normal plasma (mixing study) and (ii) use of a high-phospholipid reagent; adequate shortening of the clotting time with high phospholipid (considered a confirmatory test) but not with the mixing study supports a lupus anticoagulant. In contrast, correction by normal plasma and not high phospholipid is supportive of a common-pathway factor deficiency (although mixing studies may correct prolongations by weak lupus anticoagulants).

C. Testing of samples from patients who are receiving anticoagulants is contraindicated or requires test modifications, depending on the type of anticoagulant.

X. Whole blood clotting times

A. Point-of-care (POC) whole blood analyzers: These can measure PT, PTT, or [Fbg] for certain species. Because the samples are whole blood, high or low sample Hct (e.g., < 25 % or > 55 %) that may affect results may not be apparent. Similarly, without centrifugation to harvest plasma, hemolysis from traumatic venipuncture may not be detected to warn of potentially inaccurate results because of activation of coagulation during collection. Handling should be done in a way to prevent or limit foaminess that may interfere with testing. Manufacturer recommendations should be followed for sample testing, including storage and use of test cartridges or strips. RIs may be markedly different from those of other methods, particularly for PTT.

1. Coag Dx Analyzer and SCA2000 Veterinary Coagulation Analyzer: These analyzers measure PT (dogs, cats, horses) and PTT (dogs, cats) by optical detection of decreased fluid movement as a clot forms.
2. CoaguChek XS: This handheld analyzer designed to monitor warfarin therapy in people measures PT in a drop (as little as 8 μL) of fresh (not anticoagulated) blood. Blood reacts with recombinant human thromboplastin in the test strip to initiate the TF pathway. Thrombin generation is measured electrochemically, not via clot formation. PT is reported in seconds and as an INR, although human INR values are not appropriate for other species. Results are not displayed for PT < 9.6 s, which occurs with some healthy dogs. Unexplained prolongations have been reported in dogs.
3. VETSCAN VSpro: This on-site optical analyzer uses 1–2 drops of citrated anticoagulated whole blood to measure PT and PTT in dogs and cats; it also measures clottable [Fbg] in horses. Capillary flow moves the blood sample into a cartridge, where it mixes with dry reagents. Samples with RBC agglutination should not be used.

B. Activated coagulation (clotting) time (ACT): POC whole blood screening tests of the surface-induced (intrinsic) and common pathways

1. Several ACT methods are used in human medicine, largely for monitoring heparinization during surgical procedures. In veterinary medicine, visual (manual) tests

have been used for decades, with automated POC methods becoming available more recently.

2. Principle and method of visual tests: Nonanticoagulated whole blood is added to a tube containing particulate contact activators of the surface-induced (intrinsic) pathway. The blood is mixed, incubated, and checked every 5 s or every 10 s for the first evidence of a clot. The time from contact with the activator to initial clot formation is the *ACT*, reported in seconds to the nearest 5 or 10 s, depending on the frequency of inspection.
   a. Historically, Becton Dickinson vacuum tubes were used for this test, and publications documented their use and RIs, but those tubes are no longer sold. They contained diatomaceous (siliceous) earth, which is fossilized silica-rich diatoms (one brand of which is referred to as Celite).
   b. Several companies have developed their own versions of ACT tubes and tests using a variety of single or mixed activators (kaolin, diatomaceous earth, glass beads). Variations in these product guidelines include different blood volumes, different activators, and different recommendations for sample mixing, incubation temperature, timing of clot inspections, and recognition of the endpoint. Published reports of the diagnostic performance for these tests were not found; few reference guidelines have been published.[180,181]
3. Automated POC ACT testing
   a. The i-STAT POC analyzer can be used with ACT cartridges. ACT is measured by amperometric detection of substrate cleavage by thrombin rather than by detection of clot formation. Results for dogs, cats, and horses are compared to provided RIs. The provided upper reference limit for dogs is nearly identical to that obtained in an independent study, which also showed that the ACT decreased substantially with just brief delays in testing samples from healthy dogs.[182]
   b. Other POC analyzers measure ACT using the same photo-optical clot detection methods that are used for PT and PTT. Manufacturer recommendations should be followed.
4. Interpretive considerations
   a. A prolonged ACT indicates decreased in vitro function of the surface-induced (intrinsic) or common pathways and is interpreted similarly to a prolonged PTT (see Table 5.6 and Coagulation and Anticoagulation, Section III). However, the test may require > 90–95 % deficiency of a single factor for a prolonged result.
   b. Severe thrombocytopenia (< 10×10^3 platelets/μL) is commonly stated to prolong ACT because of decreased phospholipid availability; the assay relies on the blood sample to provide the needed phospholipid for coagulation. However, with the historical Becton Dickinson tubes, ACT values could be WRI with severe thrombocytopenia, and moderate thrombocytopenia had no effect.[183,184]
   c. Traumatic venipuncture and delayed or traumatic transfer of blood from syringe to the currently-used nonvacuum tubes should be avoided.
   d. Assay temperature: Incubation at a controlled temperature of 37 °C is generally recommended and is provided by POC analyzers. With the visual method, tube incubation in the tester's axillary area has been advocated by some and addressed in one comparison study that found "reasonable" agreement with incubation in a heating block,[185] but it was not clear if axillary temperatures were measured through thin or thick clothing or directly against the skin, and the actual axillary temperatures at the time of each test were not measured. Axillary temperatures

through clothing may vary substantially with changes in environment and clothing, and enzyme activity is significantly affected by temperature.

C. Modified Lee–White whole blood clotting time:[186,187] Although rarely used in veterinary medicine, this insensitive method of screening for defects in the surface-induced (intrinsic) and common pathways could have value in certain settings when money and time are limiting. Nonanticoagulated whole blood is tested immediately after collection, coagulation being initiated by the glass tube.
   1. Protocols require multiple analyses, standardization of blood volume, clean glass test tubes of a standard size, and a water bath (25–37 °C). Several modifications of the method have been used.[188] Variations in protocol, including venipuncture technique, blood volume, glass tubes size and coating, temperature, and sample Hct, influence the results.
   2. Reported reference values are 4–15 min for horses and cattle, 3–13 min for dogs, and about 8 min for cats.[186] Longer times (8–21 min) were found in a group of nine healthy dogs.[189]
   3. When confounding variables are controlled, prolonged times indicate severe defects in the surface-induced (intrinsic) and/or common pathways, including those caused by coagulation inhibitors.
   4. In studies assessing whole blood clotting time and PTT to detect small amounts of FIX activity in hemophiliac dogs, prolongations required < 1–2 % FIX activity; as little as 0.01 % FIX activity was enough to shorten the clotting time to less than 60 min.[190] Shortening of the prolonged PTTs required about 10× as much FIX, but still only 0.1 % of the FIX activity in healthy dogs.

XI. Endogenous anticoagulants

A. Antithrombin (AT), also known as antithrombin III (ATIII), is usually assessed by chromogenic (functional) assays rather than immunoassays that detect AT antigenically without assessing function. AT is assessed for decreased activity, which may cause or be caused by a procoagulant state.
   1. Principle and method of the routine functional test: Test plasma is added to a diluting reagent containing heparin, excess thrombin or FXa (depending on the assay), and the corresponding chromogen-labeled substrate for thrombin or FXa. Increased AT in the test plasma causes less activity of thrombin or FXa and therefore less color change (measured spectrophotometrically).
   2. Units: Units are % activity compared to species-specific (or human) plasma pool considered to have 100 % activity (also reported in decimal form, i.e., 0.8 instead of 80 %). Because of species differences in AT activity, reference and reported values vary with the species used for the reference pool.[191–193] AT also has been reported in U/mL or U/dL, where 1 U/mL or 100 U/dL, respectively, is assigned as the mean value of the reference sample pool.
   3. Stability: AT appears to be stable in citrated plasma for 6 mo at –70 °C and for 6 wk in whole blood stored at 4 °C.[193]
   4. Interpretive considerations
      a. Foals and human neonates have been shown to have plasma AT activities that are substantially lower than adult values.[129,194] This must be considered when interpreting AT values in young patients when age-matched RIs are lacking.
      b. AT activity may be overestimated when measured by some thrombin chromogenic assays because heparin cofactor II activity may be detected in addition to AT activity.[193,195]

5. Decreased AT activity may be either a cause or an effect of hypercoagulable and therefore prothrombotic states. Other clinical and laboratory findings should be used to help determine its pathogenesis (Figure 5.4 and Table 5.10).
   a. Decreased AT production
      (1) Inherited deficiencies have not been reported in domestic mammals but occur in people as type I (decreased antigen and activity, a quantitative disorder) and type II (decreased activity but normal amounts of antigen, a qualitative disorder).
      (2) Hepatic disease (including portosystemic shunts)[196] may cause or contribute to AT deficiency in several ways, including decreased AT production related to decreased functional hepatocellular mass.
      (3) Inflammation decreases AT production in some species; AT was shown to be a negative acute-phase protein in human hepatic cells and in baboons.[197] Although postulated to be a positive acute-phase protein in cats, that has not been proven, and AT activity is commonly decreased in cats with inflammatory disease, whether from consumption, hepatic disease, loss, or a negative acute-phase response.[198] It is difficult to differentiate causes of decreased AT activity in clinical cases.
      (4) Estrogens may contribute to AT deficiency by causing a mild to moderate decrease in AT synthesis.[194,199]
      (5) Prednisone administration (2.0–22.5 mg/kg daily) to healthy dogs for 14 d was associated with mild to moderate decrements in AT activity, possibly caused by decreased AT production.[200]
   b. AT loss: Like the similarly sized albumin molecule, AT may be lost from the body in animals with protein-losing nephropathy, protein-losing enteropathy, or severe

Table 5.10. Disorders or conditions that cause decreased antithrombin (AT) activity

Decreased production
Hereditary (not reported in domestic mammals)
Acquired
Hepatic disease*
Inflammation (possibly, in some species)
Hyperestrogenism
Possibly glucocorticoid therapy
Loss
Protein-losing nephropathy*
Protein-losing enteropathy
Severe hemorrhage
Consumption: increased hepatic clearance of AT–enzyme complexes
Localized coagulation (e.g., hemorrhagic trauma) or thrombosis
DIC*
Sepsis*
Heparin administration*
Pancreatitis (consumption via trypsin–AT complexes)

* A relatively common disorder, condition, or mechanism
Note: Neonates may have lower AT values than adults.

blood loss.[201] Urinary loss of AT may contribute to thrombotic disease in nephrotic syndrome patients, though other factors are involved.[202,203]

c. Increased AT consumption (increased hepatic clearance of AT–enzyme complexes)
   (1) Localized coagulation or disseminated consumptive coagulation in prothrombotic disorders (see Thrombosis, Section II) may cause decreased plasma AT activities when hepatic clearance of AT–enzyme complexes exceeds AT production.[130,196,204,205] However, significant thrombus formation may occur without decreased AT activity.
   (2) Heparin therapy (unfractionated and LMWH) accelerates the use and hepatic clearance of AT leading to decreased plasma activity.[206,207] Because AT is required for heparin's full anticoagulant effects, patients with subnormal AT activities are expected to have diminished responses to heparin and may be heparin resistant.[208]
   (3) Although pancreatitis is a prothrombotic condition that may cause consumption of AT complexed to coagulation enzymes, hepatocyte clearance of trypsin–AT complexes may also cause or contribute to decreased AT activity.[209–211]

6. Increased AT activity: AT activity is not clinically assessed to detect increases but increases may occur.
   a. Exogenous cortisol administration to dogs was associated with mildly to moderately increased AT synthesis.[199] Increased[212] and decreased[213] AT activity have been reported in dogs with hyperadrenocorticism.
   b. Cats that were made taurine deficient had greater plasma AT activities than they had prior to being fed a taurine-deficient diet,[214] and cats with cardiac disease or hyperthyroidism had increased AT activities.[76,215] AT activity decreased after radioactive iodine treatment of hyperthyroid cats, as did [Fbg] and VWF:Ag, both of which were increased in most cats prior to therapy.[76] Thyroid hormones may promote increased hepatic synthesis of AT.[76,216]
   c. Dogs with diabetes mellitus may have increased AT activity.[217]
7. TAT form whenever thrombin is generated in the presence of AT and can be measured to assess for activation of coagulation, especially latent coagulation that is not otherwise apparent. This has been done primarily for research purposes. Equine and canine plasma [TAT] has been measured with a human ELISA.[213,218–221] An automated chemiluminescent immunoassay for human [TAT] appeared to detect canine TAT based on findings in dogs with or without evidence of DIC.[222] In this assay, antibody-coated magnetic particles capture TAT and then enzyme-tagged secondary antibodies react with a substrate to emit light. A POC analyzer's chemiluminescent enzyme immunoassay for TAT has undergone initial evaluation for use in dogs.[223] Biases among assays may be substantial.
   a. Increased plasma [TAT], which may occur without an appreciable decrease in AT activity, indicates an increase in local or systemic thrombin generation and therefore thromboembolic disease or a hypercoagulable state. Decreased hepatic clearance of TAT could theoretically contribute to increased plasma [TAT].
   b. TAT may readily form in vitro if collection techniques are poor and cause coagulation.[224]
   c. Units: µg/L or ng/mL

B. Protein C activity is typically measured to detect decreases, which may cause or be caused by a procoagulant state. It is assessed by functional chromogenic[225] or clotting[226] assays that measure the protein C anticoagulant potential by activating the protein C in a sample

and assessing the resultant activity. This assay measures protein C activity but does not measure APC that was already present in a patient. Immunoassays can be used to measure protein C concentration antigenically, but these assays cannot detect functional deficiencies such as those induced by vitamin K antagonism or deficiency (protein C is vitamin K dependent).[227,228] Antigenic assays may, however, be used to differentiate type I (quantitative) from type II (qualitative) congenital deficiencies. Modifications of human protein C assays are required for domestic mammals.[229,230]

1. Chromogenic assay: Protein C in test plasma is activated by addition of a protein C activator, typically one derived from the southern copperhead snake (*Agkistrodon contortrix contortrix*). Protein C then acts on a chromogenic substrate to yield a change in light absorbance over time which is compared to a standard curve. For dogs, the curve is typically based on pooled plasma from healthy dogs and results are provided as % activity relative to that pool considered to have 100 % activity. Because the chromogenic substrate interacts with only the active site of APC, chromogenic assays may not detect rare defects in protein C that impair binding to thrombin, endothelial protein C receptor, protein S, or phospholipid.
2. Clot-based assay: This is usually a modification of the PTT assay. Human protein C-deficient plasma is added to diluted test plasma to maintain an excess of all coagulation factors such that when a protein C activator is added, the resulting prolongation of the PTT reflects only the anticoagulant effects of patient protein C (inactivation of FVa and FVIIIa) and is not caused by an underlying factor deficiency. Protein C activity is directly proportional to the PTT prolongation. Results may be reported in seconds or as a percentage of pooled plasma considered to have 100 % activity.
3. Decreased protein C activity (Table 5.11) (not to be confused with decreased APC in vivo; in vitro protein C activity could be decreased when there is increased APC in vivo)
   a. Decreased or defective production

Table 5.11. Disorders or conditions that cause decreased protein C activity

Decreased or defective production
Inherited deficiency, quantitative or qualitative (rare)
Hepatic disease (cirrhosis, hepatic failure, chronic hepatitis)*
Vitamin K antagonism or deficiency*
Portosystemic shunts (decreased hepatoportal perfusion)*
Increased consumption
Prothrombotic or hypercoagulable disorders including DIC, sepsis, recent invasive surgery, and congestive heart disease (see Thrombosis section)*
Acute traumatic coagulopathy
Certain envenomations (anticoagulant coagulopathy)
Other or multiple mechanisms
Aflatoxicosis

* A relatively common disorder, condition, or mechanism

Note: Protein C activity is greater in neonatal foals than in adult horses; age-related differences in other species are likely. Clot-based tests may yield falsely increased values with unfractionated heparinization, anti-Xa and anti-IIa oral anticoagulants, or lupus anticoagulants; they could yield falsely decreased values with factor V mutations or marked increases in FVIII activity.

(1) Hereditary deficiencies (type I, antigenic; or type II, functional): These are rarely identified in domestic mammals. A qualitative (type II) deficiency was diagnosed in a 2-yr-old thoroughbred colt with renal disease and recurrent venous thrombosis.[231] A 4-yr-old Hungarian vizsla with ventricular thrombosis had a persistently low protein C activity with no other demonstrable cause.[232]
(2) Hepatic disease with decreased functional hepatic mass: Disorders include cirrhosis, hepatic failure, and chronic hepatitis.[233]
(3) Vitamin K antagonism or deficiency: With a deficiency of reduced vitamin K in hepatocytes, protein C carboxylation and therefore function is impaired.
(4) Portosystemic vascular anomalies:[233,234] Reduced hepatoportal blood flow is likely responsible for decreased synthesis of functional protein C. Protein C activity increases with successful shunt occlusion. In contrast, few dogs with microvascular dysplasia had decreased protein C activity.

b. Increased protein C consumption
(1) Prothrombotic or hypercoagulable states (see Thrombosis, Section II) may increase activation and subsequent protease removal of protein C at a rate greater than the rate of protein C synthesis, making decreased protein C activity a marker for such states. Notable conditions include DIC and sepsis.[235,236] Decreased protein C activity in dogs with congestive heart failure appeared to relate to a prothrombotic state.
(2) *Acute traumatic coagulopathy* (*acute coagulopathy of trauma and shock*) is the pre-therapy impairment of hemostasis occurring when severe physical trauma causes vascular damage, shock, and hypoperfusion leading to an anticoagulative and hyperfibrinolytic state. Mechanisms are thought to include (i) increased activation of protein C by thrombin–thrombomodulin complexes, (ii) release of heparan sulfate, (iii) platelet dysfunction, and (iv) release of t-PA.[237] Findings in dogs with spontaneous hemoperitoneum from malignant intra-abdominal neoplasms suggested a similar protein C–consumptive disorder causing decreased protein C activity.[238,239]
(3) Certain envenomations: Some snake venoms contain a protein C activator that leads to protein C consumption and a coagulopathy.

c. Other
(1) The cause of decreased protein C activities in dogs with aflatoxicosis, including in survivors weeks after intoxication, may have been multifactorial and related to decreased functional hepatocytes, increased consumption, or uncharacterized effects of the toxin.[240]
(2) With clotting assays, false increases can occur with unfractionated heparinization, direct oral anticoagulation (anti-FXa, anti-FIIa), and lupus anticoagulants that target protein S, protein C, or FV.[230,241,242] False decreases can occur with marked increases in FVIII or with FV mutations that impair FV degradation by protein C.

d. As equine data indicate, protein C activity (not antigen) values in healthy neonates (< 24 h old) may be greater than RIs established for adults.[129,130]

4. A related test yields what is called an *APC ratio*, the ratio of the PTT measured with added APC to the PTT determined without APC being added to a patient sample.[226] A low APC ratio indicates APC resistance, the most common cause of which in people is a mutation in the gene for FV such that APC cannot cleave it an inactivate it. This is a prothrombotic condition.

XII. Anti-FXa (anti-Xa) activity:[141,226] This assay is used to measure and monitor the anticoagulant activity of heparin or LMWHs (e.g., dalteparin, enoxaparin) in plasma. Unfractionated heparin catalyzes the AT-mediated inhibition of all the coagulation enzymes, most importantly factors IIa and Xa, but its effects on thrombin require large heparin molecules that can bridge AT and thrombin together. LMWHs are enriched in smaller heparin molecules that cannot simultaneously bind to AT and thrombin, so inhibition of thrombin is reduced, and PTT assays are insensitive to their effects. In contrast, all the molecules in LMWH products promote the AT-mediated inhibition of FXa, so inhibition of FXa activity can be used to monitor their effects.

A. Principle and method
   1. Bovine FXa is added to a mixture of diluted patient plasma plus a chromogenic FXa substrate to hydrolyze the substrate and yield a product that can be measured by absorbance. Any heparin (unfractionated or LMWH) in the patient sample works with patient AT to inhibit FXa. Therefore, the amount of product generated is proportional to FXa activity and inversely proportional to anti-FXa (heparin) activity (not concentration) in the sample (i.e., more heparin causes less signal). In a modification of the assay (a 2-stage assay), AT is added to remove effects of AT deficiency.
   2. Unfractionated heparin or specific LMWH calibrators are used to generate a standard curve relating the results to heparin or LMWH activity.
   3. Units: IU/mL, usually reported to the nearest 0.1 IU/mL; "IU" refers to international pharmacologic unit of measure for heparin activity
   4. The same basic assay can be adapted for use with some other inhibitors. For example, anti-FXa activity of rivaroxaban, a nonheparin, nonwarfarin direct FXa inhibitor, can be assessed with use of rivaroxaban calibrators; results are reported in units of ng/mL and indicate the concentration of rivaroxaban associated with the measured anti-FXa activity. PTT is not suitable for monitoring rivaroxaban, and PT is less sensitive and not specific for rivaroxaban anticoagulation.

B. Interpretive considerations
   1. Targets for therapeutic peak anti-FXa heparin activities are currently based on human recommendations, which vary and may or may not be appropriate for a particular species, heparin product, or anti-FXa assay.
   2. If a patient has AT deficiency, anti-FXa results reflect impairment of heparin's function because of the decreased AT activity (unless a 2-stage assay is used). PTT assays are less affected by decreased AT activity, but they are less accurate monitors of heparinization.[243]
   3. Because the assay is colorimetric, lipemia, marked hyperbilirubinemia, and moderate or marked hemolysis may interfere with the results; consult specific laboratory and assay information.

XIII. Coagulation factor antibodies (inhibitors): Antibodies to coagulation factors are commonly referred to as *inhibitors* because they can inhibit coagulation and predispose patients to hemorrhage. They may form spontaneously as part of an autoimmune disorder (autoantibodies)[244] or develop in response to repeated transfusions in hemophiliacs who develop alloantibodies to the deficient factor.[245–247] They must be differentiated from endogenous physiologic anticoagulants, exogenous anticoagulants, and antiphospholipid antibodies. The following process can be used to detect suspected coagulation factor antibodies.

A. Coagulation factor antibodies prolong whichever coagulation times are dependent on the target factor, so unexplained prolongations of PTT, PT, or both (usually PTT) should prompt consideration of relevant deficiencies and inhibitors. When PTT is prolonged without PT prolongation, unfractionated heparin should be considered a potential cause. Resampling, analysis with a heparin inactivator, or evaluation of TT (prolonged with heparin) could be considered.

B. If heparin cannot be incriminated, the coagulation times should be determined by use of mixing studies that assess the effects of diluting the sample 1:1 with platelet-free pooled plasma from healthy individuals of the same species. Coagulation times should be assessed immediately and after a 1–2 h incubation at 37 °C because some anti-FVIII antibodies are time and/or temperature dependent.
   1. Immediate correction of the coagulation time, with no prolongation upon further incubation, supports a factor deficiency (including those caused by vitamin K antagonism) rather than a coagulation factor antibody. Normal pooled plasma should supply enough factors to correct a prolonged coagulation time. Factor analysis can be done to identify the deficient factor(s).
   2. Failure to correct the coagulation time supports the presence of a coagulation factor antibody, an antiphospholipid antibody (lupus anticoagulant), or an exogenous anticoagulant if not already excluded. However, some inhibitors may be present but at low enough concentrations for the target factor in the added pooled plasma to correct the coagulation time.
   3. Immediate correction with inhibition after incubation supports a coagulation factor antibody that had a delayed effect (some human anti-FVIII antibodies catalyze the hydrolysis of FVIII).

C. If mixing studies suggest an antibody, specialized tests can be done to assess for the presence of antiphospholipid antibodies (see next section). To further assess and characterize coagulation factor antibodies, mixing studies of test plasma with factor-replete plasma (e.g., species-specific pooled plasma) can be used in specific factor assays to determine the inhibitor strength.[244]

XIV. Antiphospholipid antibodies

A. Rarely assessed or reported in veterinary medicine,[248,249] *antiphospholipid antibodies* are IgG, IgM, or IgA molecules produced in a variety of disease states, including autoimmune and infectious disorders, that target one of the following:
   1. Proteins that bind to negatively charged phospholipids (cardiolipin, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine)
   2. Protein–phospholipid complexes
   3. Negatively charged phospholipids themselves

B. Certain antiphospholipid antibodies cause thromboembolic disease (antiphospholipid syndrome). In people, these are typically IgG or IgM isotypes that target cardiolipin and cell-bound β2-glycoprotein I or prothrombin; they may interfere with endogenous anticoagulants, inhibit fibrinolysis, activate complement, or induce prothrombotic effects on endothelial cells, platelets, and monocytes.

C. Antiphospholipid antibodies are detected by immunoassays or, in some cases, by their functional effects on coagulation tests, both of which are needed to meet criteria for the diagnosis of human antiphospholipid syndrome.

D. *Lupus anticoagulants* are a subset of antiphospholipid antibodies that inhibit phospholipid-dependent coagulation tests (e.g., PTT and PT), thus prolonging times if

the test plasma is sufficiently devoid of phospholipid. Despite the name, they are not restricted to patients with lupus erythematosus, and they are not anticoagulants in vivo where they actually promote thrombosis.

1. Basic laboratory support for the presence of a lupus anticoagulant as the cause of a prolonged PTT or PT involves exclusion of other coagulopathies, showing correction of the prolonged time by increasing phospholipid in the test system, and failure to correct the prolonged time when the test plasma is mixed with an equal volume of species-specific, pooled platelet-poor plasma (correction supports a factor deficiency but could occur with a weak lupus anticoagulant).
2. The dRVVT, a phospholipid-dependent test that is not affected by intrinsic-pathway factors or antibodies to them, can be used to demonstrate the presence of a lupus anticoagulant when addition of phospholipid overcomes the impaired coagulation. Details of the recommended procedures for human samples, including testing in anticoagulated patients, are described elsewhere.[250]

FIBRIN(OGEN)OLYSIS

I. Physiologic processes
   A. *Fibrinolysis* is the enzymatic degradation of fibrin. It counteracts coagulation and helps restore normal vessel architecture and patency after hemorrhage has been controlled with a secondary hemostatic plug. *Fibrinogenolysis* is the enzymatic degradation of Fbg. However, "fibrinolysis" is often used to refer generally to states in which both may be occurring, i.e., *fibrin(ogen)olysis*. Increased fibrinolysis is expected concurrently with prothrombotic states, in which case it is considered secondary fibrinolysis. Primary fibrinolysis is caused by defects in the fibrinolytic system, such as deficiencies in α_2-antiplasmin, PAI-1, or FXIII. Hyperfibrinolytic states promote premature breakdown of thrombi and therefore hemorrhage. Dogs are relatively hyperfibrinolytic as a species.
   B. The fibrinolytic system, like the coagulation system, is a complex network of zymogens, enzymes, activators, and inhibitors (Table 5.12). Few tests of the fibrinolytic system have broad clinical use. VET, which includes assessment of fibrinolysis, is discussed in a separate section of this chapter.
   C. Fibrinolysis is initiated simultaneously with coagulation and is normally localized to the hemostatic plug (Figure 5.1C).
      1. Locally released t-PA and u-PA cleave fibrin-bound plasminogen to form plasmin, the major fibrinolytic enzyme. Plasmin degrades fibrin and Fbg to form FDPs (Figure 5.6), which can be measured to assess fibrin(ogen)olysis.
      2. Plasmin-mediated cleavage of fibrin also exposes more binding sites for tPA and plasminogen, thus promoting further fibrinolysis. Plasmin also degrades cofactors Va and VIIIa, VWF, HK, and other prothrombotic factors.
      3. The fibrinolytic system is controlled mostly by PAI-1 (inhibits t-PA and u-PA), α_2-antiplasmin (inhibits plasmin), and TAFIa (inhibits plasminogen and t-PA binding to fibrin). Most plasmin released into circulation is rapidly inhibited by α_2-antiplasmin and cleared by the liver. PAI-1 is inactivated by the anticoagulant APC, thus promoting fibrinolysis.
   D. FDPs appear to be cleared from circulation primarily by the liver (hepatocytes and macrophages).[253–255] Nonhepatic parts of the MPS may also be involved.[256,257] The smallest D-dimer fragments (DDE, Figure 5.6) are too large ($M_r \cong 240{,}000$) to pass through an

Table 5.12. Fibrinolytic factors and their major functions

Factor[a]	Function
α_2-antiplasmin	Binds, inhibits, and clears plasmin from circulation
α_2-Macroglobulin	Binds, inhibits, and clears plasmin from circulation
D-dimers	FDPs of fibrin that contain D-dimer moieties generated by FXIIIa-mediated cross-linking; includes high and low molecular weight products
FDPs	Fragments of Fbg or fibrin produced from enzymatic degradation (fibrinolysis or fibrinogenolysis); inhibit platelets, thrombin, and fibrin polymerization
Fibrin	Major protein in a stable thrombus; source of FDPs
Fbg (FI)	Required for coagulation to progress to fibrin; converted to fibrin via thrombin (FIIa)
PAI-1	Inhibits fibrinolysis by inactivating t-PA and u-PA; produced by endothelial cells
Plasmin	Major enzyme that breaks down fibrin; also degrades Fbg and other proteins
Plasminogen	Inactive plasma protein precursor of plasmin; becomes localized to thrombi by binding to fibrin and Fbg
TAFI	Zymogen activated by thrombin and thrombomodulin to TAFIa, which cleaves plasminogen-binding sites from fibrin, thus inhibiting fibrinolysis
t-PA	Converts plasminogen to plasmin; important in fibrinolysis within the vasculature
u-PA	Converts plasminogen to plasmin; important in tissue remodeling

[a] The nomenclature follows recommendations made jointly by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis and the Committee (Commission) on Quantities and Units (in Clinical Chemistry) of the International Federation of Clinical Chemistry (IFCC) and the International Union of Pure and Applied Chemistry (IUPAC).[251]

intact glomerular filtration barrier,[258] and renal excretion does not appear to play a significant role for the smallest Fbg degradation products ($M_r \cong 60{,}000$).[259]

II. Fibrin or fibrinogen degradation products (FDPs) (fibrin fragments + Fbg fragments), also called fibrin split products
   A. Purpose: The measurement of FDPs (D-dimers are a subset of FDPs) is primarily used to detect increased fibrinolysis) associated with excessive coagulation. It also may be used to detect increased primary hyperfibrin(ogen)olysis.
      1. *Primary hyperfibrin(ogen)olysis* is a term used to describe increased fibrin(ogen)olysis because of excessive production or release of t-PA or reduced plasmin inhibitors (PAI, α_2-antiplasmin, α_2-macroglobulin).
      2. *Secondary hyperfibrin(ogen)olysis* is a term used to describe increased fibrin(ogen)olysis secondary to stimulated coagulation, as in consumptive coagulation.

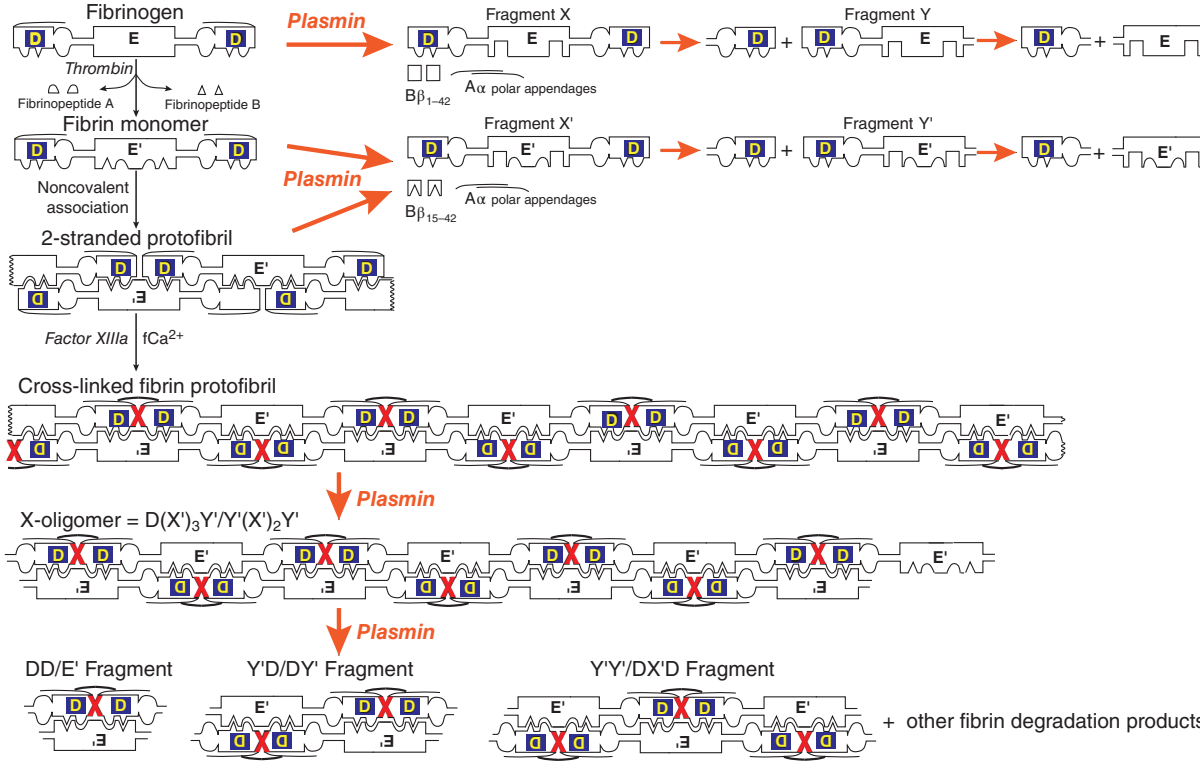

Fibrinogen
Plasmin
Fragment X
Fragment Y
Thrombin
Fibrinopeptide A
Fibrinopeptide B
Bβ1–42
Aα polar appendages
Fibrin monomer
Fragment X'
Fragment Y'
Plasmin
Noncovalent association
Bβ15–42
Aα polar appendages
2-stranded protofibril
Factor XIIIa
fCa2+
Cross-linked fibrin protofibril
Plasmin
X-oligomer = D(X')3Y'/Y'(X')2Y'
Plasmin
DD/E' Fragment
Y'D/DY' Fragment
Y'Y'/DX'D Fragment
+ other fibrin degradation products

Figure 5.6. Schematic representation of cross-linked fibrin formation from Fbg (*thin arrows*) and of the plasmin-mediated degradation of Fbg and fibrin to form FDPs (*orange arrows*).

- Fibrin formation: Fibrin is formed from Fbg, an elongated molecule that has a central E region and peripheral D regions (named for presence in the D and E fractions of FDPs eluted from an ion-exchange column). When thrombin is generated, it cleaves fibrinopeptides A and B from the E region of Fbg to form fibrin monomers. Unlike Fbg, fibrin monomers can polymerize (noncovalently) to form hydrogen-bonded protofibrils of two or more strands that are twisted around each other (two are shown here, but not twisted). Thrombin also activates FXIII to FXIIIa, which cross-links adjacent D regions of different fibrin monomers to form stable cross-linked fibrin protofibrils. When long enough, these can laterally aggregate and branch to form a network of fibrin fibers (not shown) and a stable clot or thrombus.
- FDP formation: Plasmin cleaves fibrin and Fbg at specific sites to form fibrin fragments + Fbg fragments, collectively referred to as fibrin and Fbg degradation products (FDPs).
  - Fbg: This is cleaved to form fragment X, and smaller fragments referred to as $B\beta_{1–42}$ and Aα polar appendages. Fragment X is further degraded to fragments D and Y, and fragment Y is degraded to fragments D and E.
  - Non–cross-linked fibrin: Note that the degradation of non–cross-linked fibrin is similar, differing only because of the removal of fibrinopeptides A and B from Fbg during the formation of fibrin. Fibrin monomers and non–cross-linked protofibrils are degraded to form fragment X′ and the smaller fragments $B\beta_{15–42}$ and Aα polar appendages. Fragment X′ is further degraded to fragments D and Y′, and fragment Y′ is degraded to fragments D and E′.
  - Cross-linked fibrin: Plasmin-mediated degradation of cross-linked fibrin produces a different set of FDPs because of the covalent bonds formed by FXIIIa between adjacent D regions. Initial degradation of cross-linked fibrin yields X-oligomers, high molecular weight compounds containing series of end-to-end X′ segments. These may be further degraded to other FDPs, including fragments DD/E′ (the major terminal breakdown product), Y′D/DY′, and Y′Y′/DX′D.[252]
  - Antigenic similarities of FDPs produced from Fbg and fibrin make the FDP assay nonspecific for fibrinogenolysis or fibrinolysis (with or without cross-linking). D-dimer assays are meant to detect only those FDPs containing D-dimer units indicative of fibrinolysis following coagulation.

B. Sample: serum or plasma
   1. Serum assays: Blood is collected into a special tube containing thrombin or *Botrox atrox* venom (reptilase), either of which can be used to consume Fbg via coagulation. This is done because the assay uses a polyclonal antibody to FDPs that also binds Fbg, so Fbg must be depleted. Reptilase has enzymatic activity similar to thrombin but is resistant to AT inhibition and not affected by heparin or direct thrombin inhibitors. Soybean trypsin inhibitor or aprotinin are also present in the tube and inhibit plasmin and therefore in vitro FDP formation.
   2. Plasma assays: Plasma assays use a monoclonal antibody to FDPs that does not react with Fbg, thus allowing the use of citrated plasma (which contains Fbg) as the test sample. Blood is collected as for screening coagulation tests (see Coagulation and Anticoagulation, Section II). As with other tests of hemostasis, care must be taken to prevent or minimize coagulation during collection and processing. When plasma is the test sample, clotted samples should be discarded.
   3. Stability: It is often recommended to harvest plasma promptly and test plasma within 24 h when it is stored at 4 °C, 8 h when stored at room temperature, and within 20 d when it is frozen at −20 °C. Consult your laboratory.

C. Analytical concepts
   1. Latex agglutination immunoassays are used.[260] Dilutions of test serum or plasma are incubated with latex beads coated with antibodies to human FDPs. Agglutination of the beads indicates an increased [FDP]. Although measurement of [FDP] has been done for cavitary fluid supernatants, validation studies for this sample type are lacking.
   2. Antibody specificity
      a. The specific reactivity of assay antibodies with different FDP fragments from veterinary species is not known, so it is not known exactly what is being measured, but cross-reactivity of some polyclonal reagents with canine FDPs has been shown.[261] Clinical correlations with disease also support that there is at least partial cross-reactivity in dogs.
      b. Two FDP assays, one serum and one plasma, did not appear to have clinical utility in identifying fibrinolysis in horses with colic because there were positive results in healthy horses and few increased values in horses suspected of having DIC;[262] the plasma assay also did not appear to have clinical utility in cats.[263]
   3. Unit: usually µg/mL, or mg/L (SI unit); may also be reported in ng/mL or µg/L
   4. Reported semiquantitatively as the following:
      a. Less than a low decision threshold (e.g., < 5 µg/mL with plasma tests and <10 µg/mL with serum tests) as expected in health
      b. Increased (5–20 µg/mL with plasma tests and 10–40 µg/mL with serum tests)
      c. Increased more (> 20 µg/mL with plasma tests and > 40 µg/mL with serum tests)
   5. Interpretive considerations
      a. Prozone: With a high [FDP], agglutination may occur at the greater dilution but not at the lesser dilution.
      b. False results
         (1) Generation of FDPs during traumatic blood collection can cause false increases with plasma or serum assays.
         (2) Results may be false if the assay antibodies do not bind to target FDPs or if they bind to other proteins.
         (3) Serum assay results may be falsely decreased by FDP incorporation into the clot as it forms.[264]

(4) Serum assays may be falsely increased by incomplete removal of Fbg from the sample due to the presence of dysfibrinogenemia (rare) or heparin (if thrombin rather than reptilase is used to activate clotting).[265]

c. As equine data indicate, values in apparently healthy neonates may be greater than RIs established for adults.[129,130] Values in apparently healthy elderly people are often increased, probably related to changes in clearance rates or to an increased incidence of occult disease.[266] This might be true in domestic mammals.

D. Increased [FDP] occurs in numerous disorders and conditions in which at least one of the following is present (Table 5.13):

1. Increased fibrin(ogen)olysis (lysis of fibrin ± Fbg)

a. Localized intravascular coagulation with mostly fibrinolysis (e.g., isolated thrombosis or thromboembolism, hemostasis following surgery) is a cause of secondary fibrin(ogen)olysis (see Thrombosis section).

b. DIC, nonovert or overt, also causes secondary fibrin(ogen)olysis. There are many causes of DIC (see Consumptive coagulopathies in Section II.C of Major Bleeding Disorders, and also the Thrombosis section). Clinically recognizable thrombi or associated signs may be absent with an increased [FDP] when the prothrombotic state is low-grade.

c. Hemorrhage: Increased [FDP] and [D-dimer] have been detected in the serum or plasma of dogs with hemorrhage, but values were usually not as great as those in dogs classified as having thromboembolic disease or DIC.[267,268] The reparative thrombotic response to vascular defects causing hemorrhage may generate enough FDPs through secondary fibrin(ogen)olysis to cause an increased [FDP], particularly with widespread hemorrhage. Alternatively, FDPs formed by plasmin activity in body cavities after internal hemorrhage might return to blood via lymphatics.[269] A hyperfibrin(ogen)olytic syndrome secondary to spontaneous hemoperitoneum and similar to acute traumatic coagulopathy has been suggested.[238] However,

Table 5.13. Disorders or conditions that cause increased concentrations of FDPs[a]

Increased fibrinolysis and/or fibrinogenolysis
Localized intravascular coagulation (isolated thrombosis and vessel repair)
DIC and underlying prothrombotic states including certain cardiovascular disorders, severe inflammation, sepsis, canine immune-mediated hemolytic anemia, severe tissue trauma, neoplasms, protein-losing nephropathy, and certain hepatic disorders (see text)*
Hemorrhage
Cavitary effusions, hemorrhagic and nonhemorrhagic (e.g., congestive heart failure)
Excessive release of t-PA: hypotensive shock, surgical trauma, heatstroke, acute traumatic coagulopathy and angiostrongylosis
Venom-induced consumptive coagulopathy, fibrinolytic envenomations
Administration of plasminogen activators
Decreased FDP clearance
Hepatic disease

* A relatively common disorder, condition, or mechanism

[a] FDPs include D-dimers, but increased fibrinogenolysis alone does not increase [D-dimer].

increased serum [FDP] was not detected in dogs when hemorrhage into tissue was experimentally mimicked by pumping blood from the jugular vein to the peritoneal cavity or to muscle.[270]

d. Fibrin(ogen)olytic activity in nonhemorrhagic cavitary effusions may contribute fibrinolytic activity or FDPs to plasma, e.g., via lymphatics, thus increasing the plasma [FDP] and [D-dimer] directly or indirectly.[271,272] A hyperfibrinogenolytic state related to fibrinolytic activity in cavitary spaces was suggested in part because of an increased prevalence of "discordant" plasma FDP and D-dimer results in dogs with effusions compared to healthy dogs and sick dogs without effusions; "discordant" results were defined as increased [FDP] without a concurrent increase in [D-dimer] (or a relatively greater increase in [FDP]). However, the analytical sensitivities and specificities of the assays and their respective antibodies for various Fbg and fibrin degradation products in domestic mammals are unknown, so "discordant" results of FDP and D-dimer assays may relate to differences in detected molecules and potentially different effects of an effusion matrix on FDP and D-dimer assays.

e. Excessive endothelial release of t-PA can cause primary hyperfibrinolysis. Implicated disorders include hypotensive shock, surgical trauma, acute traumatic coagulopathy (see Coagulation and Anticoagulation, Section XI.B.3.b(2)), heatstroke, and possibly some cases of angiostrongylosis.[239,273,274]

f. Certain envenomations contain factors that induce fibrinolysis,[275] and others cause VICC with thrombin-like enzymes that degrade Fbg and fibrin without activating FXIII to form cross-linked fibrin.

g. Administration of plasminogen activators (e.g., t-PA, u-PA, or streptokinase) promotes fibrinogenolysis and fibrinolysis in existing thrombi.[276]

h. Greyhounds with delayed postoperative hemorrhage are suspected of being hyperfibrinolytic; reports of plasma [FDP] were not found.

2. Decreased FDP clearance

a. Decreased hepatic clearance may contribute to the increased [FDP] in some patients with hepatic disease.[255] However, hyperfibrinolysis without DIC has been suspected to occur in some dogs with acute or chronic hepatopathies,[274] and that might cause increased [FDP].

b. Strong evidence for a renal role in clearance of FDPs, including D-dimers, is lacking. Canine experimental studies suggested that decreased renal catabolism (not decreased excretion) might contribute to increased [FDP] in some patients with renal failure,[259] but reported increases in patients with renal disease could relate to increased FDP production.

c. Theoretically, decreased MPS activity could contribute to increased [FDP], but documentation was not found.

E. Effects of increased [FDP] on hemostatic functions and tests: An increased [FDP] promotes an antithrombotic (prohemorrhagic) state and prolongation of clotting times (PT, PTT, TT, ACT, and Lee–White). If there is a marked increase in [FDP] concurrent with hypofibrinogenemia, measurements of [Fbg] may be falsely decreased (falsely prolonged TT_{Clauss}). Platelet function is also impaired.

1. Coagulation is impaired because FDPs compete with Fbg for the active site of thrombin and thus can inhibit the conversion of Fbg to fibrin.[277] Also, FDPs associate with fibrin monomers and may disrupt normal polymerization.[161,278]

2. Platelet function may be impaired because FDPs compete with Fbg for platelet binding sites, thus inhibiting platelet aggregation.[279]

III. D-dimers (upper case D is the standard label for specific regions of the Fbg molecule)
  A. Theory: D-dimer assessment can be used to detect increased fibrinolysis associated with coagulation. It should not detect fibrinogenolysis because antibodies should react only with fibrin cross-linked by the action of FXIIIa (Figure 5.6). However, the spectrum of antigenic targets of the various available assay antibodies has not been well characterized for domestic mammals.
  B. Sample
    1. The usual sample is citrated plasma collected and handled as described for FDP analysis and coagulation assays. As with the FDP assay, when serum samples are used, in vitro D-dimer formation must be prevented, and D-dimer fragments may be lost into the clot. Although [D-dimer] has been measured in cavitary fluid supernatants, validation studies for this sample type were not found.
    2. Stability: It is recommended to test plasma within 24 h when stored at 4 °C and within 20 d when frozen at −20 °C.[280] Samples from 2 of 30 healthy dogs changed from negative to mildly increased after storage at −25 °C for 12 mo; positive samples did not change from being positive when stored at −25 °C and retested anywhere from 2 to 11 mo later, but numeric data were not provided.[281]
  C. Analytical concepts
    1. The immunoassays and antibodies currently used to detect D-dimer fragments in domestic mammals were developed for human D-dimers. Immunoturbidimetric, ELISA, card immunofiltration, and semiquantitative microparticle agglutination tests have been evaluated for veterinary use, and their diagnostic utility has varied with the species, assay method, sample type, and antibody used.[262,280–284] Standardization and universal calibrators are lacking; human standards are used for veterinary samples.
    2. Antibody specificity: Most kits use monoclonal antibodies to human D-dimers that do not react with human Fbg, Fbg fragment D, or other FDPs lacking D-dimer domains.[285] There are many human D-dimer assays for central laboratory use and many others for POC use.
      a. Although the assay name suggests that only terminal D-dimer fragments are measured, assays detect D-dimer domains on a spectrum of human fibrin compounds.[285] These include high molecular weight cross-linked fibrin complexes, fibrin X-oligomers, and fibrin fragment DD/E′ (Figure 5.6).
        (1) Antibodies vary in reactivity, some preferentially reacting with high molecular weight fibrin oligomers, and others preferentially binding to low molecular weight D-dimer compounds.
        (2) D-dimer assays are specific for fibrin formation, but antibodies in some kits bind human D-dimer domains without plasmin-induced degradation.[286] Some antibodies detect D-dimer moieties produced by cathepsin or elastase activities, not just plasmin-mediated fibrinolysis.
      b. Target molecules detected by D-dimer test antibodies in veterinary samples are mostly not characterized, so the specificity of the test antibodies for D-dimer domains of veterinary species has not been proven, i.e., other molecules including fibrin(ogen) fragments without D-dimer units might be detected. One monoclonal antibody for human D-dimers showed moderate reactivity with canine D-dimers without significant Fbg binding in an assay system, but binding to Fbg degradation products was not tested.[287] Despite uncharacterized binding, certain assays appear to have value based on clinical correlations in patients suspected of having or not having DIC or prothrombotic states; other assays do not.[263] Supportive of

diagnostic value is that a POC immunochromatographic assay with a monoclonal antibody for canine D-dimers (not currently available) compared well to ELISA and latex agglutination assays with anti–human-D-dimer antibodies.[267]

3. Unit: Reporting units vary and include mg/mL, μg/mL, ng/mL, and μg/L provided in terms of either D-dimer units (D-DU), based on the mass of single D-dimer units, or Fbg equivalent units (FEU), based on the Fbg mass (1.75 × D-dimer mass)[288] from which D-dimers arose; when comparing values, RIs, and decision limits, one must be sure that the units align.

D. Increased plasma [D-dimer]: Interpretation is similar to that for increased [FDP] (Table 5.13), except that values theoretically reflect only increased cross-linked fibrin formation and fibrinolysis (not fibrinogenolysis) or decreased clearance of fibrin degradation products (not Fbg degradation products) by the liver or MPS.[289,290]

1. In human medicine, D-dimer assays have been most useful in excluding clinically significant thrombotic disease when testing patients with low to moderate pretest probability of venous thromboembolism; thrombosis is rare in such patients when [D-dimer] is not increased. However, latex agglutination assays may not detect mild increases detectable with other assays and therefore are less useful in excluding thrombotic disease in people.[288]
2. Increases in [D-dimer] can occur without overt DIC or thrombotic disease when an animal is in a prothrombotic state (see Thrombosis), e.g., with inflammation, with hemorrhage, or after surgery.[268] Postoperative increases in [D-dimer] were reported to be similar in greyhounds with and without postoperative hemorrhage that is thought to reflect a hyperfibrinolytic state, but FDP concentrations have not been reported.[291]
   a. Testing serial sample dilutions of up to 1:8 and using higher decision thresholds increases the diagnostic specificity of results for clinically significant thrombotic or thromboembolic disease.[268]
   b. Diagnostic specificity for thromboembolic disease was 94 % in one canine study using a decision threshold of 1000 ng/mL and including postsurgery dogs and dogs with hepatic disease, renal disease, cardiac disease, and neoplasms. Diagnostic sensitivity was 80 % with this decision threshold, so a negative result could not exclude thromboembolic disease.
3. Unlike in dogs and people, healthy horses have a detectable [D-dimer] when current assays are used. Detectable concentrations are therefore abnormal only when exceeding a defined decision threshold (e.g., > 1000 ng/mL).[262]

IV. Other assays may be available to measure fibrinolytic components (e.g., plasminogen, α_2-antiplasmin, plasmin inhibition, t-PA, PAI-1, TAFI) (Table 5.12) or fibrinolytic activity (euglobulin lysis time test, plasma kinetic turbidometric assay),[292] but their diagnostic utility is limited or unknown and they are not widely used in clinical veterinary medicine. VET can assess fibrinolysis and is discussed in the following section.

GLOBAL ASSESSMENT OF HEMOSTASIS: VISCOELASTIC TESTING (VET)

I. Definitions

A. *Viscosity* can be thought of as "thickness" (e.g., syrup is more viscous than water), but it is defined as resistance to deformation (i.e., cold syrup resists moving out of squeezed bottle while water flows out more freely). Blood viscosity increases with increasing Hct or protein concentration and decreases with anemia.

B. *Elasticity* is the tendency for a material to return to its original state when subjected to a distorting influence; as blood clots, it becomes more elastic.
C. *Viscoelastic properties* are the characteristics of a material that give it viscosity and elasticity. Blood is a viscoelastic fluid (mostly viscous properties), and fibrin clots and thrombi have viscoelastic properties that can vary with the thrombus location (e.g., arterial vs venous), the cellular and protein content, the thrombus age, and even by region within a single thrombus. These variations may affect whether a thrombus breaks and embolizes, occludes a vessel, or responds to thrombolytic therapy.[293] Less-stiff thrombi may be more deformed by flowing blood (allowing blood past), whereas more-elastic thrombi may return to their original shape after deformation. Stiffer thrombi are more likely to break and embolize.
D. *Shear stress* is shear force per unit area applied to a material (SI unit: Pa; also dyn/cm^2); *shear force* promotes movement of one part of a material relative to other parts of the material, and in the same plane as the applied force.
E. *Shear strain* is distortion of a material by movement of parallel internal components past each other in response to a shear stress; it is a unitless ratio of the change in length to original length.
F. *Shear modulus* is the ratio of shear stress to shear strain (SI unit: Pa; also dyn/cm^2); a large shear modulus means that a large stress is required to cause displacement (the material is stiff), while a small modulus reflects a less-stiff material.

II. General Concepts
A. In the context of hemostasis, *viscoelastic testing (VET)* is the kinetic assessment of the viscoelastic properties of whole blood or plasma as blood clots and potentially as a formed clot continues through lysis. It has been considered a global assessment of hemostasis because all the elements of blood, cellular and soluble, are present and contribute to clot formation. In comparison to classical coagulation tests, VET assesses more than just the initiation of coagulation and may better assess prothrombotic states by better reflecting the balance between procoagulant factors on the one hand and anticoagulants and fibrinolytic factors on the other. However, the testing cup surface and induction of mechanical stress may not mirror the in vivo environment, and the important effects of endothelial cells (e.g., thrombomodulin-mediated APC and TAFI generation or thrombin removal, VWF release), blood flow, and removal and replenishment of various substances involved in the hemostatic process are not assessed by VET. Also, testing is done under shear stress conditions that are lower than in arteries and many veins and venules.
B. The simultaneous assessment of the net effects of many contributors to hemostasis is both a strength (assessing net effect) and a weakness (may not pinpoint a cause for abnormal hemostasis) of standard VET.[294]
C. First described in 1948, standard VET began as a research tool that was later developed as a relatively rapid POC assessment of human patients at high risk for massive hemorrhage and transfusion management, i.e., cardiovascular surgery, liver transplantation, trauma, and postpartum hemorrhage. There has also been extensive evaluation of VET for many other uses, including detection of prothrombotic states and hyperfibrinolysis in sepsis, states not assessed well by classical hemostasis testing. Modifications of standard VET through use of activators and inhibitors has yielded tests with more analytical specificity.
D. However, lack of standardization, the need for site-specific RIs, and use of open systems with manual pipetting has led to centralization of some VET testing and spurred development of cartridge-based VET instruments with more automation, repeatability, and reliability for POC use.

III. Major VET instruments (Note: Results and RIs are not interchangeable among analyzers or for different test methods used with one analyzer.)
   A. Thrombelastography (now often called thromboelastography, TEG)[295,296]
      1. To use the TEG 5000 Thrombelastograph Hemostasis Analyzer, a 340 μL sample of whole blood (or plasma) is added to 20 μL of a $CaCl_2$ solution (± other reagents) in a heated test cup which is then rotated back and forth (2.375° to each side of neutral) in 10-second cycles with pauses before each reversal of direction. A so-called pin extending into the sample within the cup is attached to a torsion wire above. As the cup rotates prior to sample clotting, the pin does not turn, but as fibrin forms between the cup and the pin, the pin is pulled along with the cup and turns back and forth as the cup rotates. In the original TEG analyzer, a small mirror on the pin reflected light from a slit lamp onto a moving roll of photographic film where it generated a graphic image, the *thrombelastogram*: reflection from the stationary pin generated a single straight line before clotting, and the line diverged with clot formation as the light reflecting from the rotating pin exposed the film during each 1-second pause at maximal rotation in each direction. In modern TEG analyzers, an electromechanical sensor detects pin rotation and generates an analogous graph (Figure 5.7).
      2. The TEG 6s is a more-automated cartridge-based system requiring only 20 μL of citrated whole blood. It assesses sample shear modulus by proportional changes in resonance frequency as the blood clots. The meniscus of a droplet of blood suspended by surface tension at the bottom of a narrow ring (short cylinder) bulges below the lower rim of the ring. A light-emitting diode directs a light beam toward the bulging droplet (meniscus), creating an optical shadow on a light sensor. The droplet is stimulated to vibrate over a range of ultrasonic frequencies, inducing motion in the sample meniscus, which resonates at its resonant frequency. The light sensor measures displacement of the droplet, and the frequency of vibration is calculated throughout clotting and lysis of the sample, increasing as the clot binds to the ring and clot stiffness increases. Frequency outputs include tabular and graphical representations of clot strength converted to millimeters, similar to the TEG 5000. This method eliminates potential mechanical effects of pin and cup contact on blood coagulation. Results of the TEG 6s and TEG 5000 are not interchangeable.[297]
   B. Thromboelastometry (ROTEM)
      1. Kinetic assessment of clotting by the ROTEM delta Thromboelastometry system is similar to that by TEG, but the heated cup is stationary in ROTEM, and it is the pin inserted into the blood sample that rotates through 4.75°. With clotting, pin movement is restricted in inverse proportion to the strength of the forming clot, and pin movement is measured by a photosensor that detects reflection of a collimated light beam off a mirror coupled to the pin (similar to the original TEG apparatus). The graphic representation (TEMogram) is similar to that of TEG (Figure 5.7). Because of methodologic differences including cup composition, sample volume (321 μL), and reagent types and amounts, ROTEM and TEG results differ.
      2. The ROTEM sigma system is an automated cartridge-based system that eliminates pipetting and maintains the pin and cup method. Cartridges containing lyophilized reagents withdraw blood from a tube and distribute it to cups in separate channels that run different tests simultaneously.
   C. Sonoclot Coagulation & Platelet Function Analyzer: This microviscometer is a linear-motion system rather than a rotational-based system. A vertically oscillating hollow plastic

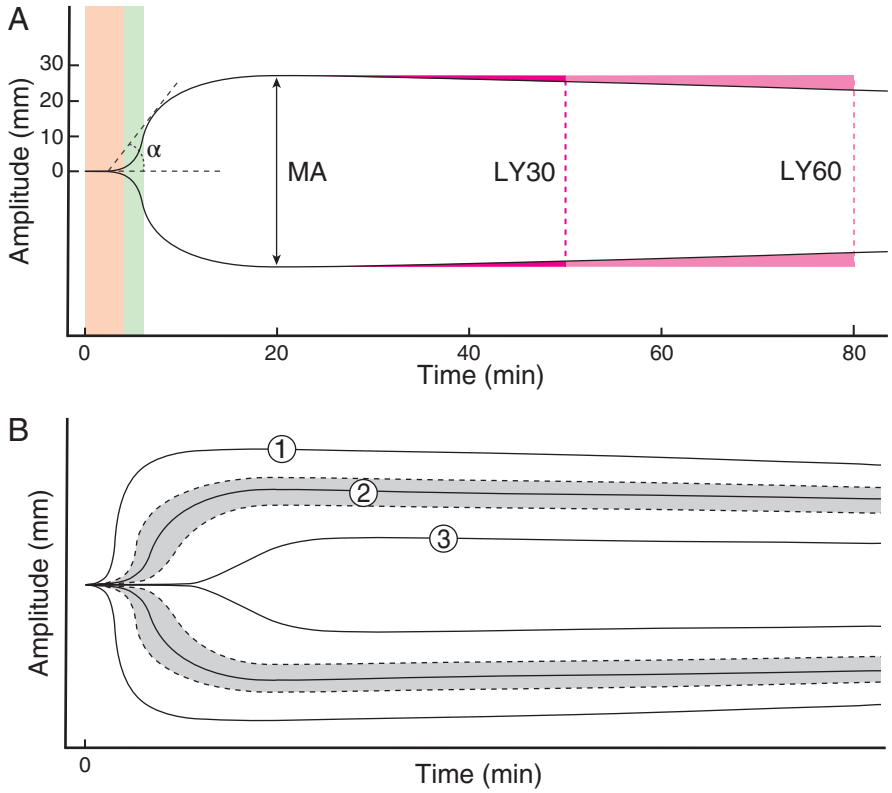


Figure 5.7. TEG curves in health and hypo- or hypercoagulable states. Thrombelastographs begin with a horizontal line that diverges into two symmetrical curves as a sample clots. The time to clot initiation, the rate of clot formation, the strength or stiffness of the clot, and clot lysis are reflected by the shape of the curve and the numeric data generated. See Table 5.14 for definitions of each parameter illustrated here.

A. This thrombelastograph for a typical healthy dog illustrates R (*tan interval*), K (*green interval*), α (*blue angle*), and MA. LY30 is calculated as the percentage decrease in area under the curve occurring in the 30 min after reaching MA; the reduction is shown in *dark pink*. For the LY60 calculation, the reduced area caused by a decreasing amplitude is the sum of the *dark and light pink areas*. In health, curve shape and associated values vary with the specific method and clot initiator. Little (shown here) or no clot lysis is seen in health.

B. Thrombelastograph curves illustrating common changes seen with ① hypercoagulable (prothrombotic) and ③ hypocoagulable (prohemorrhagic) samples compared to ② health. The grey zone schematically indicates variations in health.

① Hypercoagulable sample: R and K are decreased; α and MA are increased. LY30 and/or LY60 could be increased if there were increased secondary fibrinolysis (not shown).

③ Hypocoagulable sample: R and K are increased; α and MA are decreased. With marked hypocoagulability, the baseline may not diverge at all (e.g., heparinized sample without heparinase). K cannot be determined if divergence does not reach 20 mm.

Note: ROTEM yields similar types of curves (TEMograms) with similar hyper- and hypocoagulable changes, but terms (Table 5.14), specific data, and RIs are different.

probe immersed in the sample of native blood or citrated blood plus $CaCl_2$ is connected to a transducer that transforms changes in probe oscillation as clotting occurs to yield a graphical signature. The greater the sample resistance to probe motion, the greater the graphed output signal, which is normalized to reference standards and provided in so-called clot signal units. Kits are provided for either kaolin or glass bead activation.

D. VCM Vet: This small and very portable analyzer assesses viscoelastic properties of untreated whole blood (340 µL) that enters a cassette by capillary action and passes between two parallel glass discs that activate coagulation via the surface-induced pathway. One of the discs is rotated and one is free. As a clot forms between the discs, the free disc begins to move with the powered disc and the movements are translated into a graph and measurements similar to the output of other VET analyzers. Samples should be tested within 4 min of collection.

E. Other methods of VET are under development.[295] In addition, nonVET global assessments of hemostasis include thrombin generation by calibrated automated thrombography (CAT) and fibrin generation assays (overall hemostatic potential, OHP).[298]

IV. Analytical concepts of VET

A. Because VET may be used in POC settings by operators who may not already be knowledgeable of good laboratory procedures, it is important to note that all VET operators should be appropriately trained and should adhere to manufacturer recommendations and protocols, including quality assurance and quality control procedures. VET analyzers should not be placed where they may be subject to movement or substantial vibrations. Serial patient samples should be run on the same specific analyzer because of analyzer variability (affects some measurands more than others).[299]

B. Sample: The usual sample for ROTEM and TEG is 3.2 % citrated whole blood, collected with care as described for classical hemostasis testing (see Coagulation and Anticoagulation, Section II). Standardization of all sample and sampling variables helps reduce preanalytical error and variations in results.

1. It is not clear if use of 3.8 % citrate tubes in place of 3.2 % tubes yields clinically significant differences in ill patients, but errors related to underfilling tubes (excess citrate, impaired coagulation) are reportedly greater with use of 3.8 % citrate tubes, so these are not recommended.[300]
2. Sampling method should match what was used for RIs unless another collection method has been shown to yield the same results in an array of healthy and diseased individuals of the species of interest and tested with the same specific method.
   a. Atraumatic jugular collection with vacuum tubes is an appropriate method in animals that have large enough jugular veins, but other veins or a catheter may be used. Consistent "clean" technique is particularly important for VET without use of activators, as such results can be affected by venipuncture quality, vacuum versus syringe collection, and collection site.[301] However, sampling method can also significantly impact activated VET, so consistent sampling is always best practice.[299]
   b. If transferring blood from syringe to multiple tubes, which is not the recommended approach, the citrate tube for VET should follow any serum tube and precede any tubes containing other anticoagulants.
3. Nonanticoagulated whole blood can be tested within about 5 min of collection, but this is not practical when testing is centralized to a laboratory, and it requires RIs generated with similar samples and the same protocol.

4. Plasma harvested from citrated whole blood is used for certain nonstandard tests. Centrifugation at high force may remove enough platelets and microparticles to prevent native citrated plasma coagulation.

C. Sample processing
   1. For TEG and ROTEM, a standard rest interval of 30 min between collection and testing has been recommended because of mild, progressive increases or decreases in coagulability, most prominent when activators are not used.[302–306] A consistent rest interval for patient and RI samples is appropriate.
   2. Room temperature storage is generally recommended.[302] Most information supports that samples should not be refrigerated, and storage at 37 °C has been done but may yield mildly different results.[305,307]

D. Activation: For all anticoagulated samples, a standard amount of Ca^{2+} is added during analysis to allow coagulation to occur, but additional reagents may be used to activate the surface-induced (intrinsic), TF (extrinsic), or both pathways. Activators are recommended to override variations in coagulation caused by sample collection and processing variables.[301,302] They do so by increasing the rate of thrombin generation, accelerating coagulation and potentially increasing clot strength,[307,308] but this may also mask certain pathologic abnormalities, such as hypercoagulable states or AT deficiency. Multiple sample aliquots tested with a variety of activators (and inhibitors) may be needed to adequately characterize certain abnormalities;[302] monoanalysis (use of one method) is less likely to reveal a particular abnormality or its cause.[294] Results and appropriate RIs vary with each method.[308] The standard TEG and ROTEM tests and activators currently used in veterinary medicine are as follows:
   1. Native TEG or NATEM: This is use of a citrated sample with calcium but no added activator. Activation occurs through the contact pathway as coagulation factors are exposed to the test cup and pin. Native TEG is sensitive to preanalytical variations in sample collection and processing.
   2. Kaolin-activated TEG: Kaolin is hydrated aluminum silicate. Proprietary kaolin-coated tubes accelerate activation of the surface-induced (intrinsic) pathway via FXII and can be used with or without heparinase. Heparinase, an enzyme from *Flavobacterium heparinum*, rapidly cleaves heparin into inactive fragments and can be used to determine either (i) the effects of heparin in the patient, or (ii) the patient's hemostatic state with minimized heparin interference.
   3. RapidTEG reagent: Coagulation is more rapidly initiated through surface-induced (intrinsic) and TF (extrinsic) pathways by a proprietary combination of kaolin and TF reconstituted from lyophilized powder.
   4. TF: Although a selective TF reagent is not available from the Haemonetics (TEG) company, TF is commonly used at various concentrations by diluting commercially available TF reagents for the PT test. Innovan has been recommended for use at dilutions of 1:3600 in horses and also at 1:50,000 in dogs and cats.[302]
      a. Results, particularly clotting time, differ with different TF concentrations.
      b. Use of Innovan may promote errors related to reagent storage, product variations, and the required large dilutions that magnify pipetting errors.
   5. INTEM (intrinsic TEM): For ROTEM, proprietary ellagic acid is used with phospholipids to activate the surface-induced (intrinsic) pathway. HEPTEM incorporates heparinase with ellagic acid.
   6. EXTEM (extrinsic TEM): For ROTEM, proprietary recombinant TF activates the TF (extrinsic) pathway.

E. Assessed phases of the hemostatic process: Data provided by TEG, ROTEM, and the VCM Vet are similar, reflecting the rate of clot initiation and formation, clot strength, and potentially clot lysis (Table 5.14). Similar visual representations of the data are generated as clotting occurs to yield thrombelastogram and TEMogram patterns (Figure 5.7) that

Table 5.14. Definitions and components of basic ROTEM and TEG parameters

Assessment	Parameter		Unit	What is measured
Clot initiation				
TEG	R	Reaction time	min	Time from start to 2 mm amplitude[a]
ROTEM	CT	Clotting time	s	Time from start to 2 mm amplitude[a]
Clotting rate				
TEG	K[b]	Kinetic time	min	Time from 2 mm to 20 mm amplitude
	α	Angle	°	Angle between the baseline and a tangent to the curve passing though one of several points[bc]
ROTEM	CFT	Clot formation time	s	Time from 2 mm to 20 mm amplitude
	α	Angle	°	Angle between the baseline and the tangent to the curve that passes through the 2 mm amplitude point
	CFR	Clot formation rate	°	Angle between baseline and the tangent to the curve at the maximal slope
Clot strength				
TEG	MA	Max. amplitude	mm	Maximum curve amplitude reported as the distance between diverging lines
ROTEM	MCF	Max. clot firmness	mm	Maximum curve amplitude, reported as the distance from baseline to a curve
Fibrinolysis				
TEG	LY30 LY60	Lysis index 30 Lysis index 60	%	Percentage ↓ in area between curves 30 or 60 min after MA compared to no change in amplitude after the MA
ROTEM	LI30 LI45 LI60	Lysis index 30 Lysis index 45 Lysis index 60	% % %	Amplitude at 30, 45, or 60 min after CT endpoint (2 mm amplitude) as a % of MCF (e.g., LI30 = A30/MCF × 100)

[a] Amplitude (A) is the vertical distance between diverging curves for TEG but only the vertical distance from center midline to upper curve for ROTEM.

[b] K is undefined if an amplitude of 20 mm is not reached.

[c] The TEG 5000 default for α is a tangent through the split point, but it can also be from the point on the curve where R is reached.[294] For TEG 6s, the angle is formed by the line tangent to the curve midway between the boundaries for K.

supplement the quantitative data. However, the methods differ, and the results are not interchangeable. Sonoclot data appear different but also characterize all phases of coagulation.

1. Clot initiation: The time from the start of the test to the development of a gel with measurable stiffness is the R (reaction) time (TEG), CT (ROTEM, VCM Vet), ACT (RapidTEG), or SonACT (Sonogram). It is affected mostly by coagulation factor activities and the type and concentration of activator used to initiate coagulation. Therefore, its assessment is similar to those of standard clotting times (PT, PTT, ACT). In the absence of disease, < 10 % of the total thrombin generation and fibrin formation occur in this phase.
2. Clot formation: The rate at which the clot forms is reflected by the slope of the clot-stiffness curve and the time required to reach a given stiffness once an initial gel has formed. The former is α for TEG, ROTEM, and the VCM Vet, and clot rate (CR) for the Sonogram; the latter is K (TEG) or CFT (ROTEM, VCM Vet) and not available in hypocoagulable states if the defined amplitude is not reached. Clot formation is influenced by the concentration and function of Fbg and certain other coagulation factors, the concentration and function of platelets, and concentrations of anticoagulants. It is inversely correlated with sample Hct.
3. Clot strength: As a clot propagates, its stiffness increases as fibrin polymerizes and crosslinks to form a fibrillar network with entrapped cells. The maximal clot stiffness (shear modulus) is reported as the MA (TEG), MCF (ROTEM, VCM Vet), or peak amplitude (Sonogram). It is strongly influenced by functional Fbg and platelet concentrations, as well as FXIII activity, which makes VET a useful test for rare FXIII deficiency. It is also inversely correlated with sample Hct. Because fibrinolysis occurs concurrently with coagulation, clot strength can be affected by hyperfibrinolysis.
4. Fibrinolysis: VET samples from healthy individuals show little or no clot lysis with standard tests, but when present, lysis is indicated by a decrease in clot stiffness (curve amplitude) after maximal stiffness is reached. This can be expressed in various ways, most commonly as a clot lysis (LY) value at specific times after maximal clot stiffness for TEG, or as a lysis index (LI) value at specific times after initial clot formation for ROTEM and the VCM Vet analyzer. With hyperfibrinolysis, LY values increase but LI values decrease. Clot retraction or detachment might mimic hyperfibrinolytic patterns with some samples and analyzers.[309] With ROTEM, maximum lysis (ML) is provided as the greatest percentage decrease in amplitude from MCF; ML increases with increased fibrinolysis. Changes in the Sonoclot signature curve, including a terminal drop to or near baseline, can reflect hyperfibrinolysis.
5. Clot retraction: The Sonoclot signature pattern is affected by clot retraction, and a platelet function (PF) value from 0 through 5 is provided to characterize the quality of clot retraction based on the degree and rate of decrease in the curve from its peak.

F. Other selected data derived from standard VET measurements
   1. A frequently reported value derived from the MA or MCF is the shear modulus, *G*, calculated for the TEG as G = (5000 × MA) / (100 – MA); units, Pa/cm^2 or dyn/cm^2. This value was meant to describe the actual shear modulus created by the TEG clot, with a doubling of G being a doubling of the ratio of shear stress to shear strain (see Definitions in Section I).

a. Because G is just an arithmetic transformation of measured MA, paired MA or MCF and G values should yield the same conclusions when compared to appropriate RIs.
b. Because increases in G are essentially exponential compared to MA or MCF, arithmetic transformation of MA to G results in greater sensitivity to small changes in amplitude. However, errors and imprecision are similarly amplified, so CVs for G are high.[303]
c. The formula may be inaccurate, and its use has been questioned.[296]

2. A coagulation index (CI) can be calculated from a linear equation based on R, K, MA, and α generated by nonactivated or kaolin-activated TEG. The CI is meant to provide an overall index of a patient's coagulation status, with decreased values supporting hypocoagulability and increased values supporting hypercoagulability. Equations used in veterinary medicine should be derived from species-specific data and validated for particular uses.
3. CE (also E) is a calculated value meant to reflect clot elasticity (elasticity), and MCE is the maximum clot elasticity: MCE = (100 × MCF) / (100 – MCF), a dimensionless relative value (no units). The value is needed for certain secondary calculations, and it has been suggested that it may be more appropriate to report than G.[296]
4. A thrombodynamic potential index (TPI) is the E value of MA divided by K (or MCF), thus reflecting a combination of maximal clot strength and the time to its formation. TPI increases with shorter K (CFT) and greater MA (MCF) (considered increased coagulability).
5. Other measured and derived values reported for VET include first derivative values generated from the standard tracings to assess rates of coagulation and lysis as well as total thrombus formation and total thrombus lysis. These are beyond the scope of this book.

G. Functional Fbg assessment by TEG or ROTEM: VET parameters are assessed in paired samples tested with and without a platelet inhibitor (heparinase may be included if needed). The clot strength measured with platelet inhibition is considered the contribution of Fbg/fibrin, while the difference in results is considered an indirect reflection of the platelet contribution. Although results correlate with [Fbg], neither inhibitor fully inhibits platelets.[294]
1. TEG Functional Fibrinogen: TF activation is coupled with abciximab, Fab fragments of a chimeric human–murine monoclonal antibody that bind to the platelet Fbg receptor, $\alpha_{IIb}\beta_3$, and inhibit aggregation while not affecting platelet procoagulant expression. However, abciximab did not alter standard TEG results in dogs, and the standard TEG Functional Fibrinogen assay is not useful in dogs.[302,310]
2. ROTEM FIBTEM: Activation with TF (EXTEM reagent) is coupled with cytochalasin D, an inhibitor of actin polymerization and therefore platelet microtubule function, shape change, and procoagulant expression. The MCF generated with FIBTEM is positively correlated with clottable [Fbg],[311,312] but it is lower in dogs and cats than in people despite similar Fbg concentrations.[313] Fibrinolysis is more apparent in samples assessed by FIBTEM compared to EXTEM; FIBTEM may aide in recognition of hyperfibrinolysis.[313]

H. TEG Platelet Mapping: In standard VET, pathologic or pharmacologic platelet dysfunction is masked by generation of thrombin, a strong platelet agonist generated in response to kaolin, TF, or ellagic acid. In so-called platelet mapping, thrombin generation is suppressed by use of heparinized blood (heparin largely inhibits thrombin via AT), and

reptilase is added to convert Fbg to fibrin in the absence of thrombin. FXIIIa is also added to promote fibrin crosslinking. Concurrent assessment of clot strength with and without ADP or arachidonic acid as platelet agonists reveals the platelet contribution (% reactivity) in the absence of thrombin. Results are compared to kaolin-activated TEG, thus requiring four TEG channels (two analyzers) for full evaluation.

1. The ADP platelet mapping kit may be used in dogs without modification, but arachidonic acid is not an effective platelet agonist in a subset of dogs (dogs with thromboxane A2 insensitivity) and is therefore not appropriate for use in all dogs.[302]
2. Unmodified platelet mapping yielded highly variable results and did not show value when assessed in healthy cats, as MA did not increase above baseline with platelet agonists.[314] This may have reflected issues related to agonist concentrations or platelet activation during sample acquisition.

I. Other variations of VET include:

1. APTEM: This is EXTEM plus aprotinin to inhibit fibrinolysis, which is assessed by comparisons of paired APTEM and EXTEM data. However, in healthy cats and hyperfibrinolytic cats and dogs, APTEM tracings do not consistently show better clotting as expected if fibrinolysis is appropriately inhibited.[313,315]
2. Addition of tPA to TEG: This enhances and allows assessment of fibrinolysis, hypofibrinolysis, or hyperfibrinolysis.[308,316]
3. Addition of fresh frozen plasma in vitro to TF-activated TEG: This helps direct transfusion decisions based on the ability of plasma to improve coagulability as assessed by TEG.[316]

V. Interpretive Considerations for VET

A. Despite numerous publications describing the use and potential value of VET in human and domestic mammalian patients, many recent reviews indicate the need for prospective, controlled studies to establish clinical utility for each intended use. Many studies have characterized changes in various disorders and have shown statistically significant differences between groups, but there is often substantial overlap in results between patients with and without a particular disorder, making the statistical differences of limited diagnostic value.

B. RIs: Appropriate RIs vary with the type of analyzer, the individual analyzer, test reagents, other analytical factors such as pipetting, preanalytical variations in collection and processing, and animal populations. Because many of these variables differ among studies, it is not surprising that RIs vary.[312,317] Each VET site should establish or confirm transfer of RIs using established guidelines.[302] Sample collection, processing, and analysis should be strictly standardized for all reference and test samples. Age (immaturity), breed (e.g., greyhound),[318] and neuter status for female dogs[317] should be considered when developing RIs or interpreting results.

C. It is helpful to interpret measured quantitative VET results individually (e.g., changes in R, alpha, K, MA, and LY30), but calculated global coagulation indices such as coagulation index or thrombodynamic potential index can also be used. Contributors to increases and decreases of standard TEG and ROTEM values are summarized in Table 5.15.

1. Results should be interpreted in light of each other, the indication for testing, the activator used, all clinical findings, Hct (see later in this section), and, when available, results of standard hemostasis tests including [Fbg] and [platelet].[302]
2. In addition to quantitative assessment, the overall visual pattern (e.g., flat line, wine glass, bullet, etc.) can be very informative.

Table 5.15. Contributors to increased or decreased values for ROTEM and TEG measurands

Measurand	Increased	Decreased
R, CT[a]	↓ functional coagulation factors[b]	↑ procoagulant factors[c]
	Exogenous anticoagulants[b]	Antifibrinolytics (ε-aminocaproic acid)
	↑ endogenous anticoagulants	Decreased AT
	Thrombocytopenia (mild effect)	Thrombocythemia
K, CFT	↓ functional coagulation factors[b]	↑ procoagulant factors[c]
	Exogenous anticoagulants[b]	Hyperfibrinogenemia
	↑ endogenous anticoagulants[b]	Antifibrinolytics (ε-aminocaproic acid)
	Thrombocytopenia, certain types of platelet dysfunction	Thrombocytosis
	Erythrocytosis	Anemia
A	See causes of ↓ K and CFT	See causes of ↑ K and CFT
MA, MCF (also G)	Hyperfibrinogenemia	Hypofibrinogenemia, some dysfibrinogenemias
	Thrombocytosis (mild)	Thrombocytopenia, platelet dysfunction with some systems
	Anemia	Erythrocytosis
		Hyperfibrinolysis[d]
		Anticoagulants
		↓ functional coagulation factors[b]
		FXIII deficiency[e] (rare)
LY30[f]	↑ fibrinolytic enzymes and/or ↓ fibrinolytic inhibitors	Not detectable
	FXIII deficiency[e]	

[a] Activator/method can affect sensitivity to abnormalities, e.g., TF initiation is more affected by FVII whereas kaolin or ellagic acid initiation are more affected by FIX. However, downstream events are independent of the initiator of coagulation. R and CT reflect intrinsic-pathway initiation if no activator is used.

[b] See Tables 5.5 and 5.6 for disorders and anticoagulants.

[c] Procoagulant factors are typically not specifically identified but may include increased functional coagulation factors, TF, microparticles expressing phosphatidylserine or TF, neutrophil extracellular traps, and polyphosphates.

[d] Fibrinolysis is concurrent with coagulation, so clot strength can decrease with hyperfibrinolysis. Antifibrinolytics can help correct decreases in MA and MCF, but increases are not expected.

[e] VET is useful along with the clot lysis test to support a rare diagnosis of FXIII deficiency; standard coagulation tests are not affected by FXIII.

[f] Contributors to increased and decreased LY60 are the same as for LY30. With ROTEM, causes of increased and decreased LI30 (or LI45, LI60) are reversed from those for LY30, the latter indicating degree of lysis and the former indicating remaining clot strength.

Note: Traumatic venipuncture and in vitro hemolysis can have varied effects on VET, e.g., shortened R and K or CFT by premature initiation of coagulation, or decreased MA or MCF by inducing platelet refractoriness secondary to platelet activation.

D. Although VET is used to assess prothrombotic and prohemorrhagic states, including changes in fibrinolysis, standard VET criteria defining these states are lacking. The following changes are most commonly considered to reflect prothrombotic or prohemorrhagic states.
   1. Prothrombotic (hypercoagulable) states are supported by (i) decreased R or CT, (ii) decreased K or CFT, (iii) increased α, and/or (iv) increased MA or MCF (or their derived shear modulus G). These changes may or may not occur together; e.g., clot formation could be delayed when α and maximum clot stiffness are increased. Derived global values are also used.
      a. No values have been shown to predict clinical thrombosis in veterinary species.
      b. Hyperfibrinogenemia is present in many conditions associated with a prothrombotic (hypercoagulable) VET pattern, and while there is evidence that thrombi form more and are stronger when hyperfibrinogenemia is present, hyperfibrinogenemia alone is not thought to be a thrombus initiator.[168]
   2. Prohemorrhagic (hypocoagulable) states are supported by (i) increased R or CT, (ii) increased K or CFT, (iii) decreased α, (iv) decreased MA or MCF (or their derived shear modulus G), and/or (v) increased LY30. Derived global values are also used.
      a. No specific changes have been shown to be reliably predictive of clinical bleeding in dogs.
      b. Care must be taken to use heparinase to exclude or assess heparin effects that may be present.
   3. Hyperfibrinolysis is supported by: (i) increased LY30 (or LY60) or (ii) decreased LI30 (or LI45 or LI60). The opposite changes may reflect hypofibrinolysis.[319] Results of APTEM, FIBTEM, and tPA-modified assays are also used to identify alterations in fibrinolysis.

E. Effect of Hct on VET: Sample Hct is inversely associated with sample coagulability as assessed by TEG, thus confounding VET interpretation in patients with anemia or erythrocytosis because it is thought to be only an in vitro phenomenon.
   1. Specifically, lower Hct is associated with shorter clotting times, a greater clotting rate, and greater clot stiffness,[320,321] but this anemia-related in vitro hypercoagulability has not been associated with clinical thrombotic disease.
   2. In horses, transient phenylephrine-induced erythrocytosis without changes in [platelet] or [Fbg] yielded transient hypocoagulable changes in whole blood, but results were unchanged in plasma samples from these horses.[322] This suggests that decreased coagulability was caused by the increased proportion of cells to plasma rather a change in concentrations of citrate, Ca^{2+}, or other constituents of the fluid phase.
   3. The VET changes occurring with changes in Hct may relate to the proportion of RBCs to clotting factors, including Fbg: with a greater Hct, there is less total Fbg in a sample cup to contribute to clot size and strength, and more RBCs to potentially alter fibrin mesh formation. Note: In some studies that were designed to assess these factors, effects of manipulating Hct in vitro may have unsuspectingly altered final sample [citrate], [Ca^{2+}], or [Fbg] enough to contribute to VET changes, and centrifugation may have created microparticles promoting coagulation. In vitro changes in proportions of plasma to RBCs can affect final Ca^{2+} concentrations after standard recalcification, and final [Ca^{2+}] impacts coagulation and VET responses.

MAJOR BLEEDING DISORDERS: FINDINGS AND PATHOGENESES

I. Diagnosis: The diagnosis of bleeding disorders requires knowledge of the general types of bleeding disorders, consideration of clinical findings, accurate interpretation of hemostasis test results, and recognition of hemostasis test patterns.
   A. Types of bleeding disorders
      1. Blood vessel disorders: typically identified by nonhemostatic tests including histologic evaluation and testing for infectious diseases, but hemostatic tests may be useful to exclude primary blood disorders
         a. Large vessels: surgical or nonsurgical trauma, invasion, aneurysms, and anomalies causing gross hemorrhage
         b. Small vessels: vasculitides (infectious, immune mediated, and chemical) and vasculopathies (rare); may cause petechial and ecchymotic hemorrhage mimicking thrombocytopenia or platelet dysfunction
      2. Blood disorders: typically identified by hemostatic tests
         a. Impaired primary hemostasis: thrombocytopenia, disorders of platelet function, and VWD
         b. Impaired secondary hemostasis: hereditary or acquired defects in coagulation
         c. Excessive fibrinolysis: primary or secondary disorders
         d. Combinations of the above
   B. Clinical findings are essential for establishing a final diagnosis; bleeding pattern, breed, age, and sex may be of particular value in directing diagnostic plans.
      1. Bleeding pattern
         a. Petechiae and ecchymoses should prompt consideration of severe thrombocytopenia or a disorder of platelet function. Concurrent defects in secondary hemostasis may also be present. Vascular diseases may cause similar hemorrhages.
         b. Subcutaneous hematomas and hemorrhage into body cavities suggest defects in secondary hemostasis, especially when petechiae are absent. However, hematomas may form with platelet defects, particularly in large animals, and subcutaneous hemorrhage may occur with coagulopathies. With severe hemophilia, hematomas may develop into bone or soft-tissue hemophilic pseudotumors.
         c. Hemorrhage through mucosal surfaces (e.g., epistaxis, gastrointestinal hemorrhage, tracheal bleeding, prolonged estral bleeding, bleeding with tooth eruption) may occur with thrombocytopenia, platelet function disorders, VWD, or defects in coagulation. Prolonged hemorrhage secondary to venipuncture or surgical or nonsurgical trauma is also not specific.
         d. Bleeding into the central nervous system may be occult except for neurologic signs.
      2. Breed and age: Breed predispositions for inherited diseases may help in selection of appropriate diagnostic tests. Hemorrhage in a young animal should prompt consideration of an inherited defect of platelets (Table 5.2), VWD, or coagulation factor deficiency (Table 5.9) if another cause is not apparent. Molecular genetic testing is available for characterized mutations in some affected and carrier animals (e.g., for VWD).
      3. Sex: Hemophilia A (FVIII deficiency) and hemophilia B (FIX deficiency) are sex-linked (X chromosome), recessively inherited disorders. Therefore, these disorders almost always occur in males. Hemorrhage in females is unlikely to be caused by one of these disorders.
         a. Males are either affected or not. They cannot be just carriers.
         b. Females may be (i) free of the defect, (ii) carriers (heterozygous), or (iii) rarely affected (homozygous).

c. About 50 % of the offspring of carrier females mated to unaffected males inherit a defective X chromosome, so 50 % of male offspring are affected and 50 % of female offspring are carriers.
d. All female offspring of a clear female and an affected male are asymptomatic carriers.
e. Female offspring from a carrier female and an affected male can be affected.

C. Hemostasis tests: A summary of available screening and specialized hemostasis tests is presented in Table 5.16 to aide in selection and interpretation of specific tests.

D. Major patterns of hemostasis test results: Hemostasis in bleeding patients is best evaluated by multiple tests (hemostasis profiles). Examples of the major patterns of common hemostatic test results in bleeding patients are provided in Table 5.17.

1. Pattern 1: Prolonged BMBT without thrombocytopenia or severe anemia suggests VWD or a platelet functional defect. VWF analysis or platelet function studies may be indicated. Specifically defining disorders of platelet function may be difficult. PTT may occasionally be prolonged with canine VWD if FVIII is concurrently deficient enough, and it may be prolonged in horses with VWD.
2. Pattern 2: Prolonged BMBT associated with moderate to severe thrombocytopenia can be explained by the thrombocytopenia, though concurrent defects in platelet function or VWF cannot be excluded. However, BMBT testing is not indicated in patients with moderate to severe thrombocytopenia because prolongations of BMBT are expected and thus will not add new information.
3. Pattern 3: Isolated prolongation of the PTT indicates a surface-induced (intrinsic) pathway defect, though insensitivity of a particular PT assay to factor deficiencies could mask other abnormalities.
   a. Acquired PTT prolongations may be caused by hepatic disease, heparin contamination of the sample (e.g., inappropriate collection from a heparinized catheter), or heparin therapy, and rarely by vitamin K antagonism (PT prolongations are expected first). Rare antibodies to intrinsic-pathway coagulation factors or antiphospholipid antibodies could be considered.
   b. Inherited or congenital PTT prolongations (Table 5.9)
      (1) Hemophilia A and B caused by deficiencies of FVIII and FIX, respectively, are the most common inherited causes and may be associated with severe hemorrhage. They are extremely rare in females because they are X-linked recessive traits.
      (2) Deficiencies in FXII and PK are not associated with hemorrhage when either occurs alone. FXII deficiency is relatively common in cats, occurring in multiple breeds and more commonly than feline hemophilia.[133]
      (3) Deficiency of FXI is associated with mild hemorrhage, usually in response to surgical or nonsurgical trauma. Hemorrhage may be delayed.
      (4) Deficiencies may be multiple (e.g., FXII plus FIX deficiency).
4. Pattern 4: Isolated prolongation of PT indicates a TF pathway (FVII) defect.
   a. Acquired PT prolongations: Hepatic or hepatobiliary disease and vitamin K antagonism or deficiency should be considered. FVII has the shortest half-life of the vitamin K–dependent factors, and three of the five factors in the combined TF and common pathways are vitamin K dependent. Therefore, the PT can be affected by vitamin K antagonism or deficiency before the PTT, especially when a PIVKA-sensitive PT method is used.

Table 5.16. Possible causes of abnormal results for the major tests of hemostasis

Test or analyte	Result	Possible causes
ACT	↑ Time	Defect in surface-induced (intrinsic) and/or common pathways
AT	↓ Activity[a]	Renal or intestinal loss, consumptive coagulation, heparin administration, or decreased production
BMBT	↑ Time	Thrombocytopenia, thrombopathia, VWF deficiency, anemia
Clot retraction	↓ Extent	Thrombocytopenia, platelet dysfunction
Coagulation factors	↓ Activity	Hereditary or acquired deficiencies (hypocoagulable state)
D-dimers	↑ Concentration	Coagulation plus fibrinolysis, decreased clearance, hemorrhage, cavitary effusion
FDPs	↑ Concentration	Increased fibrin(ogen)olysis, decreased clearance, hemorrhage, cavitary effusions
Fbg[b]	↓ Concentration ↑ Concentration	Decreased hepatic production, increased consumption (may be hypocoagulable) Inflammation, hemoconcentration
Protein C	↓ Activity[c]	Decreased hepatic production, abnormal production (vitamin K antagonism or deficiency), loss, consumption
PT	↑ Time	Defect in TF and/or common pathways
PTT	↑ Time	Defect in surface-induced (intrinsic) and/or common pathways
RVVT or dRVVT	↑ Time	Defect in common pathway
TT (TCT)	↑ Time	Hypofibrinogenemia, dysfibrinogenemia, heparinized patient or sample, increased FDPs
VWF:Ag	↓ Concentration ↑ Concentration	VWD or VWD carrier Exercise, excitement, pregnancy, vasopressin

[a] Chromogenic assays assess activity, but there are immunologic assays that assess concentration. Proteins may be present but not functional.

[b] For assessment of hemostasis, Fbg concentrations are usually determined as clottable [Fbg] from TT_{Clauss} values, which are inversely correlated with Fbg concentrations unless prolonged by high concentrations of heparin or high concentrations of FDPs when Fbg concentrations are low.

[c] Chromogenic assays assess activity; immunologic assays assess concentration. A [protein C] may be WRI while activity is decreased if there is abnormal production of protein C (e.g., vitamin K antagonism or deficiency). Chromogenic assays assess only one function of protein C, not its binding to thrombin, endothelial cells, protein S, or phospholipid.

Table 5.17. Interpretation of major patterns of common hemostasis test results

Pattern	PT	PTT[a]	[FDP][b]	[Fbg][c]	[Platelet]	BMBT	Interpretation[d]
1	WRI	WRI	WRI	WRI	WRI	↑	Thrombopathia, VWD[e]
2	WRI	WRI	WRI	WRI	↓	↑	Thrombocytopenia
3	WRI	↑	WRI	WRI	WRI	WRI	Intrinsic-pathway defect
4	↑	WRI	WRI	WRI	WRI	WRI	Factor VII deficiency[f]
5	↑	↑	WRI	WRI	WRI	WRI	Common-pathway defect or multiple pathway defects
6	↑	↑	WRI	↓	WRI	WRI–↑	Dys- or afibrinogenemia, hepatic disease
7	↑	↑	↑	↓	↓	↑	Fulminant DIC[g]

[a] ACT results mirror PTT results in most disorders, but ACT prolongation requires greater factor deficiencies, so the ACT may be WRI when the PTT is prolonged.
[b] Plasma [FDP], including [D-dimer], may increase with hemorrhage (see the text).
[c] For assessment of hemostasis, Fbg concentrations are usually determined as clottable [Fbg] from TT_{Clauss} values, which are inversely correlated with Fbg concentrations.
[d] See the text (Major Bleeding Disorders, Section I.D) for expanded interpretive comments.
[e] PTT may be prolonged in horses and occasionally in dogs with VWD if FVIII is concurrently deficient.
[f] This pattern may occur with early stages of hepatic disease or vitamin K antagonism or deficiency.
[g] This pattern applies only to the severe, fulminant form of DIC.

b. Inherited or congenital PT prolongations (Table 5.9): FVII deficiencies are rare and associated with mild hemorrhage, usually in response to surgical or nonsurgical trauma.

5. Pattern 5: Prolonged PT and PTT without other screening abnormalities suggest multiple factor deficiencies or a common-pathway defect excluding afibrinogenemia or dysfibrinogenemia (clottable [Fbg] would be decreased).
   a. Acquired disorders: Combined deficiencies of vitamin K–dependent factors (II, VII, IX, and X) caused by vitamin K antagonism or deficiency may lead to severe hemorrhage. Thrombocytopenia may develop. Hepatic failure should also be considered, especially if [Fbg] is low-normal. Icterus and other evidence of obstructive cholestasis should suggest the possibility of contributing vitamin K deficiency. Variations of this pattern seen with hepatic disease include combinations of increased [FDP] and [D-dimer], decreased [Fbg], thrombocytopenia, and prolonged BMBT.
   b. Inherited or congenital disorders (Table 5.9): Rare inherited FX or FII deficiency could be considered and may be associated with severe hemorrhagic tendencies. A similar clinical and laboratory pattern could be caused by rare hereditary defects in vitamin K–dependent carboxylase (so-called vitamin K–dependent multifactor deficiency, reported in Devon rex cats and possibly found in a Labrador retriever).[323,324]
6. Pattern 6: Prolonged PT and PTT with hypofibrinogenemia and possibly prolonged BMBT
   a. Acquired disorders: Hepatic disease is a consideration for this pattern (see the discussion of pattern 5). Consumptive coagulation (e.g., DIC) could be considered, but thrombocytopenia and increased [FDP] and/or [D-dimer] are expected to be present or developing with DIC.

b. Inherited or congenital disorders: A marked decrease in [Fbg] along with prolonged PT and PTT may occur with congenital Fbg disorders including inherited dysfibrinogenemia or afibrinogenemia (rare).[21,170,171,325,326] Severe hemorrhagic tendencies are expected, and BMBT may be prolonged.

7. Pattern 7: Prolonged PT and PTT with increased [FDP], hypofibrinogenemia, and thrombocytopenia are typical of fulminant DIC, whatever the cause. Such a patient would appear very ill, likely with evidence of multiple organ damage. An underlying triggering condition for DIC should be present. Findings with hepatic failure could be identical despite lack of thrombosis or hemorrhage.

II. Pathogeneses of selected acquired bleeding disorders: The pathogeneses of hemorrhagic defects in primary hemostasis relate to specific abnormalities or deficiencies in platelets, VWF, or the vessel wall. Inherited disorders of secondary hemostasis are caused by various genetic alterations that result in decreased, absent, or abnormal hemostatic factors. The pathogenesis of acquired bleeding disorders is often more complex.

A. Hepatic disease: Disorders including cirrhosis, chronic hepatitis, acute hepatic disease, acute hepatic failure, and hepatic lipidosis can cause defects in the production and clearance of procoagulants, anticoagulants, profibrinolytics, and antifibrinolytics.[327] The net result is often relatively balanced and therefore clinically silent despite abnormalities in laboratory tests that mimic consumptive coagulation and/or consumptive coagulopathy. However, thrombosis and bleeding may occur, particularly if the hemostatic balance is tipped by a concurrent condition.

1. Potential abnormal hemostatic test results and their causes

a. Prolonged PT, PTT, ACT, or TT may occur for two major reasons. Hepatic production of coagulation factors may be decreased because of decreased functional hepatic mass (including portosystemic shunts),[328] and production of vitamin K–dependent coagulation factors may be impaired because of decreased vitamin K absorption secondary to cholestasis or, possibly, decreased food intake.

b. Decreased [Fbg], AT activity, and protein C activity may occur because of decreased hepatic production.

c. Increased [FDP] and/or [D-dimer] may occur because of decreased hepatic clearance of FDPs and plasminogen activators.

d. Thrombocytopenia may occur because of increased splenic sequestration caused by portal hypertension, splenomegaly, or possibly decreased thrombopoietin production.

e. Prolonged BMBT may occur with (i) thrombocytopenia, (ii) an acquired disorder of platelet function related to increased [FDP], or (iii) unknown factors.

f. VET results vary with the type and stage of hepatic disorder. Patterns vary from hypercoagulable to hypocoagulable in dogs with acute hepatic failure[329] or chronic hepatopathies,[330] tending toward hypocoagulability and hyperfibrinolysis with loss of hepatic function. Many VET findings correlate with standard hemostasis test results, but because PT and PTT assess only coagulation and not the net balance of hemostatic factors, VET may not suggest a hypocoagulable state when PT and/or PTT are prolonged.

2. Results of routine hemostatic tests, including VET, are not good predictors of surgical hemorrhage in human patients with hepatic disease[331] or of hemorrhage induced by collecting liver biopsy samples.[332] Hypofibrinogenemia and thrombocytopenia may be most predictive. Similarly, bleeding secondary to biopsy procedures in dogs and cats does

not appear to be predictable from PT and PTT results, although, in cats, there was an association between major complications and PTT prolongation of at least 1.5× the mean of the RI.[333] Bleeding complications in dogs and cats are more strongly associated with thrombocytopenia than with prolonged PT and PTT. In horses, there was no apparent association between hemostasis test results and complications related to liver biopsies.[334] Poor predictability of clinical bleeding from routine tests in hepatic failure is likely because the liver is responsible for synthesizing proteins used in counteracting pathways, so there is a somewhat balanced decrease in pro- and antithrombotic factors; routine tests assess the factors or pathways independently.

B. Vitamin K antagonism or deficiency
   1. Vitamin K antagonism: Bleeding is common and may be external and obvious or internal and occult. It usually occurs 3–7 d after exposure.
      a. Vitamin K antagonists may be ingested in several forms:
         (1) Hydroxycoumarins (warfarin, bromadiolone, brodifacoum, coumatetralyl, difenacoum, flocoumafen, tioclomarol) or indandiones (diphacinone, chlorophacinone, pindone) in anticoagulant rodenticide products; these are specifically detectable by high-performance liquid chromatography
         (2) Sweet clover or sweet vernal grass containing bishydroxycoumarin, a metabolite produced from the actions of certain molds[335,336]
         (3) Therapeutic coumadins: from an overdose, which could be caused by displacement from plasma proteins by additional protein-binding drugs (e.g., phenylbutazone), or by ingestion of medication intended for human patients (includes warfarin, acenocoumarol, dicoumarol, and phenoprocoumon)[337]
         (4) Excess sulfaquinoxaline, a coccidiostat with vitamin K antagonistic effects[338–340]
      b. Once absorbed, these compounds inhibit the enzymatic recycling of oxidized vitamin K back to its reduced and functional form in hepatocytes, thus leading to production of coagulation factors with decreased functional potential (Figure 5.8).[341,342] When normal coagulation factors in the circulation ($t_{1/2}$ = 6–40 h) decrease enough, coagulation times become prolonged (can occur within 24 h)[343] and hemorrhage may occur.
   2. Vitamin K deficiency (hypovitaminosis K) rarely causes hemorrhage despite prolonged PT and PTT, perhaps in part because it affects pro- and anticoagulant factors. However, vitamin K deficiency causes bleeding in human newborns and might also in domestic mammalian neonates because of low vitamin K stores, low dietary intake, and limited production by alimentary microorganisms.[344]
      a. Causes of clinically significant vitamin K deficiency have not been clarified in most species, but the following should be considered:
         1. Prolonged anorexia or ingestion of an abnormal diet may cause or contribute to vitamin K deficiency. In contrast, normal diets contain excess vitamin K.[345,346]
         2. Gut sterilization by antimicrobials may cause or contribute to vitamin K deficiency.[345,347]
         3. Malabsorption of vitamin K (a fat-soluble vitamin) may occur with intrahepatic or extrahepatic cholestasis (decreased fat digestion and absorption, and therefore decreased vitamin K absorption), intestinal malabsorptive disorders (e.g., infiltrative bowel disease), or exocrine pancreatic insufficiency (decreased fat digestion and absorption, and therefore decreased vitamin K absorption).[154]

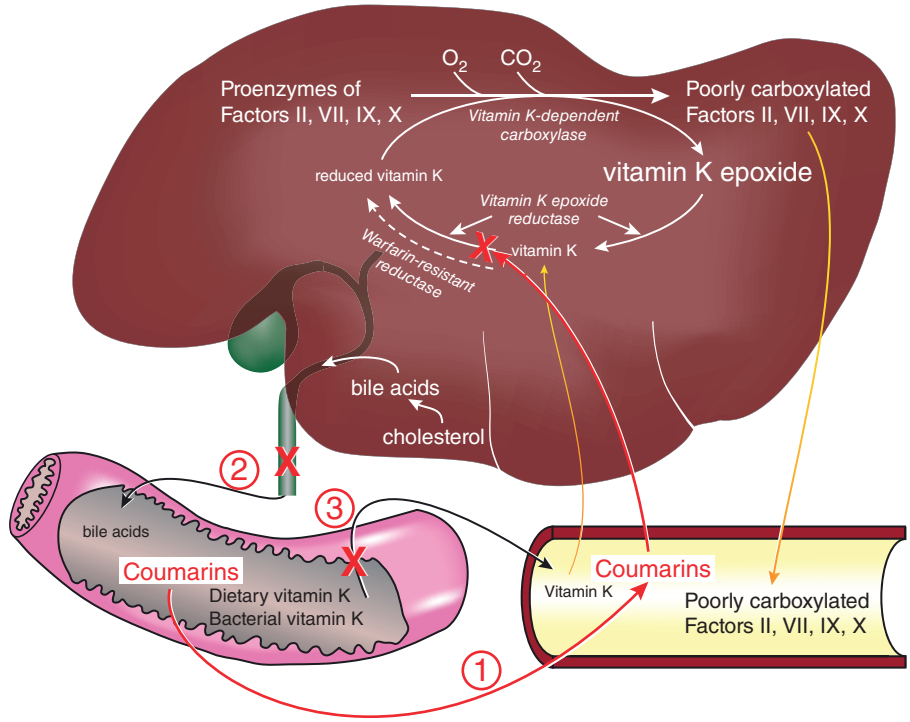


Figure 5.8. Effects of vitamin K antagonism or deficiency on hepatocyte production of vitamin K–dependent coagulation factors (II, VII, IX, and X). *Circled numbers* in the figure denote the three following processes that result in production of defective factors II, VII, IX, and X.

① Antagonism: Ingested anticoagulant rodenticides or other coumarins are absorbed in the intestine. In hepatocytes, they inhibit the enzymatic reduction of vitamin K epoxide back to reduced vitamin K, which is the form of vitamin K required for the vitamin K–dependent carboxylase enzyme to carboxylate the coagulation proteins. This appears to occur by reversibly uncoupling the first and second reductions catalyzed by vitamin K epoxide reductase. Failure to recycle vitamin K leads to decreased amounts of reduced vitamin K and increased amounts of vitamin K epoxide. Factors II, VII, IX, and X are still produced, but they enter the blood and circulate in a permanently hypofunctional or nonfunctional state. These poorly carboxylated or noncarboxylated coagulation factors are known as PIVKA (proteins induced by vitamin K antagonism) and in people can be measured with specific antibodies. Plasma vitamin K epoxide concentrations increase (not shown). Vitamin K_1 administration enables the formation of sufficient reduced vitamin K for adequate factor carboxylation; this appears to occur through the actions of a warfarin-resistant reductase rather than via vitamin K epoxide reductase.

② Deficiency because of cholestasis (intrahepatic or posthepatic): Impaired bile flow reduces fat absorption because not enough bile acids reach the intestine to emulsify ingested fats properly. Consequently, fewer fat-soluble vitamins, including vitamin K, are absorbed, and vitamin K deficiency may develop. The deficiency causes impaired carboxylation of vitamin K–dependent coagulation factors despite sufficient activity of reducing enzymes. Noncarboxylated factors are produced and enter the blood, where they are known as PIVKA (proteins induced by vitamin K absence). Plasma [vitamin K epoxide] is not increased.

③ Deficiency because of other causes: Anorexia (decreased dietary intake of vitamin K), decreased vitamin K production by intestinal bacteria (e.g., after antibiotics), intestinal malabsorptive disease, or exocrine pancreatic insufficiency can contribute to, or rarely cause, vitamin K deficiency.

b. Diminished vitamin K may lead to a subclinical mixture of PIVKA and normal coagulation factors. Vitamin K supplementation was associated with a correction of prolonged PIVKA-sensitive PT values in cats with intestinal or hepatic diseases.[154]

3. Causes of abnormal hemostatic test results
   a. Prolonged PT, PTT, or ACT
      (1) Decreased amounts of functional vitamin K–dependent coagulation factors participating in the surface-induced (FIX), TF (FVII), and common (FX and FII) pathways; factor analysis is typically not done, but deficiencies would be expected
      (2) Although PT is expected to be prolonged before PTT, there may be prolongation of PTT alone, PT alone, or both values, depending on the optimization of the assays and, perhaps, the species involved.[348]
   b. Thrombocytopenia, when present, probably results mostly from consumption at multiple sites of hemorrhage, with blood loss contributing.
   c. Other abnormalities have not been widely described, but decreased [FDP], including [D-dimer], and increased [Fbg] may result from decreased coagulation and consequently decreased fibrinolysis. Prolonged TT reported in people correlated with increases in [Fbg].[165] In contrast, however, internal hemorrhage may contribute to an increase in [FDP] and [D-dimer].
4. Rare hereditary defects in vitamin K–dependent carboxylase (vitamin K–dependent multifactor deficiency) produce similar hemostatic abnormalities, but vitamin K is neither deficient nor antagonized in this disorder.[323,324]

C. C*onsumptive coagulopathies (disseminated, localized, venom-induced)*: These are disorders of impaired coagulation caused by initial activation and subsequent consumption of coagulation enzymes. Activation may be via excessive endogenous pathways (e.g., DIC) or via exogenous enzymes that directly activate coagulation factors (VICC).[332]
1. *DIC*, *disseminated intravascular coagulation*, is an acquired syndrome characterized by excessive activation of intravascular coagulation that becomes systemic (disseminated) rather than remaining localized. Generation of thrombin and fibrin cause microvascular thrombi which can cause organ dysfunction. Excessive or unbalanced consumption of coagulation factors and platelets may lead to hemorrhage (consumptive coagulopathy), a common clinical finding. Concurrent consumption of anticoagulants (AT, protein C) and plasmin-mediated generation of FDPs and D-dimers contribute to the syndrome. It may be acute and severe (overt) or chronic and low-grade (nonovert), but it is always secondary to an underlying condition that causes unbalanced or excessive coagulation.
   a. DIC may develop from severe inflammatory states such as sepsis, endotoxemia, equine colic and colitis, systemic inflammatory response syndrome, canine immune-mediated hemolytic anemia, and acute pancreatitis. Bacterial infections are the most common septic cause, but other agents, including protozoa, can induce DIC (e.g., *Babesia rossi* in dogs or *Cytauxzoon felis* in cats). DIC may develop from various causes of severe cell damage such as blunt trauma, burns, ischemic necrosis, and heatstroke. Malignant neoplasms, particularly if metastatic, may cause DIC via several mechanisms. Some envenomations may cause DIC, but many cause VICC.
   b. Coagulation is often initiated by excessive TF released from or expressed on damaged tissues, neoplastic cells, or cytokine- or endotoxin-stimulated cells and membrane-derived microparticles from endothelial cells, WBCs, and other cells. These cells and microparticles may also express procoagulant phospholipids. When production of

procoagulant factors does not keep up with consumption, consumptive coagulopathy and fulminant DIC with hemorrhage may occur.

c. Clinical signs of consumptive coagulopathy vary from subclinical to serious hemorrhage, shock, or signs of multiple organ failures caused by thromboembolism or hemorrhage. Signs of the underlying disease may also be present.

d. Although not always the case, consumptive coagulopathy can be recognized when the typical laboratory and clinical signs accompany a condition known to trigger the process. Typical findings are thrombocytopenia, prolonged PT and PTT, increased [D-dimer] and [FDP], decreased AT activity, decreased [protein C] or, importantly, trends toward these abnormalities with serial measurements. However, combinations of these abnormalities can occur without consumptive coagulation/coagulopathy, and the patterns of laboratory abnormalities vary considerably and depend on severity, duration, and underlying or concurrent disorders.

e. Pathogeneses of the abnormal hemostatic test results

(1) Hypofibrinogenemia: Fbg is consumed as it is transformed into fibrin during excessive coagulation. Because Fbg is a positive acute-phase protein, increased hepatic production may occur in inflammatory states and mask its consumption.

(2) Thrombocytopenia: Platelets are consumed as they patch exposed vascular defects, become incorporated into thrombi, or become activated secondary to thrombin and other platelet agonists formed in the blood.

(3) Prolonged PT, PTT, and ACT: Hypofibrinogenemia, increased [FDP], and decreased functional coagulation factors may all contribute. Cofactors Va and VIIIa are inactivated by APC. Coagulation enzymes are bound by AT or other antiproteases and removed from circulation by hepatocytes. Thrombin bound to thrombomodulin on endothelial cell membranes is internalized and degraded. If the rate of enzyme inactivation and removal remains greater than the rate of production and release of new proenzymes, plasma activity of these coagulation factors decreases. Lastly, FDPs from fibrinolysis interfere with fibrin formation.

(4) Decreased AT activity: After AT binds to activated coagulation enzymes, the complexes are removed from the circulation by hepatocytes. If the rate of removal remains greater than the rate of production and release of new AT, plasma AT activity decreases.

(5) Increased [FDP] and [D-dimer]: With activation of coagulation pathways, cross-linked fibrin forms; concurrent activation of fibrinolysis causes plasmin-mediated degradation of fibrin and possibly Fbg. This leads to the increased formation of FDPs and D-dimers.

(6) Prolonged TT and TT_{Clauss}: This can be caused by hypofibrinogenemia and increased [FDP].

(7) Prolonged BMBT: This can be caused by thrombocytopenia, increased [FDP], and anemia from hemorrhage. FDPs interfere with platelet function. Anemia decreases the interaction of platelets with the vessel wall, likely from less marginalized platelet flow.

(8) RBC fragmentation: This occurs from intravascular trauma (microangiopathic fragmentation). Keratocytes and schizocytes, including spherocytic fragments, may be found in blood films. In a subset of conditions, particularly those with splenic infiltrative disease, acanthocytes may also be seen.

(9) VET results vary with the stage, severity, and other factors including [platelet], [Fbg], and Hct; patterns may appear hypercoagulable to hypocoagulable, the latter being associated with more severe clinical disease and hemorrhage.[349,350]

f. Although tissue trauma can induce DIC, severe trauma may also cause acute traumatic coagulopathy characterized by an anticoagulative and hyperfibrinolytic state without the disseminated thrombus formation and prominent consumption of platelets and coagulation factors seen in DIC (see Coagulation and Anticoagulation, Section XI.B.3.b(2)). Findings in dogs with spontaneous hemoperitoneum caused by malignant neoplasms suggested a similar state.[238]

2. *Localized consumptive coagulation*, an excessive or unbalanced coagulation that is not disseminated, is rarely extensive enough to progress to a consumptive coagulopathy and bleeding; correction of the localized problem may be curative. Human examples of localized causes of consumptive coagulopathy include aortic aneurysm and large hemangiomas. Large cutaneous and splenic hemangiomas and hemangiosarcomas may be examples of localized consumptive coagulation in dogs, but the changes have usually been interpreted as DIC.
3. *Venom-induced consumptive coagulopathy (VICC)* describes the consumption of coagulation factors and resulting impairment of coagulation caused by various procoagulant toxins introduced through certain snake envenomations.[351–354] Although laboratory findings may fulfill diagnostic criteria for DIC, making DIC a common conclusion for patients with VICC, its pathogenesis and nature differ from DIC. In VICC, the onset of coagulopathy is rapid, it resolves quickly with therapy (and sometimes without), and it often occurs without apparent fibrin deposition or resultant end-organ damage. A minority of human VICC patients develop thrombocytopenia and microangiopathic hemolytic anemia associated with acute renal failure, but the pathogenesis of this syndrome is not clear.
   a. Snake venoms vary greatly in their toxins and effects. Coagulopathic venoms are present in many vipers, many elapids, and a few colubrids, the three general types of venomous snakes. Most of these venoms include a procoagulant toxin that activates coagulation at a specific point in the pathway that varies with the toxin. Many vipers have a thrombin-like enzyme that causes hypofibrinogenemia and a relatively mild coagulopathy (without bleeding), whereas other toxins are prothrombin activators or FX activators (see dilute Russell's viper venom test) that lead to consumption of factors II, V, and VIII or factors V, VIII, and X, respectively, in addition to Fbg. Other constituents of these venoms and venoms from other animals may affect hemostasis through other mechanisms including increased fibrinolysis via plasmin-like activity or plasminogen activation, activation of other coagulation factors, increased anticoagulant activity, vascular damage, platelet activation, or platelet inhibition. Also, effects may vary with the species that is envenomated.
   b. Clinical findings of VICC most often include local evidence of a snake bite (edema, fang marks, bite-site hemorrhage), evidence of other internal or external hemorrhage (including mucosal bleeding, hematuria, ecchymoses, and petechiae), depression, tachypnea, and limping if bitten in a limb.[355,356] Venoms containing enzymes that weaken vessel walls (hemorrhagins) more often induce bite-site hemorrhage.
   c. Common laboratory abnormalities are prolongation of clotting times (PTT, ACT, and usually PT), hypofibrinogenemia, increased FDPs including D-dimers in most cases, and thrombocytopenia, thus resembling DIC. When thrombin-like enzymes predominate, [D-dimer] may be minimally increased unless the assay nonspecifically

detects other FDPs as well; this is because Fbg and fibrin are digested without activation of FXIII to form cross-linked fibrin (as occurs with endogenous thrombin generation).[351] AT and protein C activities may be decreased as well, and [TAT] may be increased.

(1) Envenomation with phospholipase-rich venoms may cause a coagulopathy that is not detected by certain PTT assays, generally those that are less affected by lupus anticoagulants.[357] Low-phospholipid reagents improve detection.

(2) Prolongations in PT caused by VICC may be missed when using the i-STAT POC analyzer or others with a similar method.[358] Instead of detecting formation of a fibrin clot, the endpoint of this assay is the thrombin-mediated conversion of an artificial thrombin substrate (not Fbg) to an electrochemically detectable product. Venom constituents interfere with this process.

d. In contrast to VICC, which is caused by procoagulant toxins, some venoms cause an anticoagulant coagulopathy (e.g., via protein C activation). Coagulation times become prolonged (especially PTT) but there is no consumption of Fbg or generation of D-dimers.[359]

D. Dilutional coagulopathy: Infusion of large volumes of colloid fluids, crystalloid fluids, plasma-poor packed RBCs, or Oxyglobin solution (a hemoglobin-based oxygen carrier) to treat massive, acute blood loss expands blood volume and transiently dilutes blood components, including coagulation factors, platelets, VWF, and Fbg.[360] This may result in prolonged coagulation times and contribute to hemorrhage, but it alone is not expected to cause hemorrhage.[361–363] It may compound acute traumatic coagulopathy. Compared to saline, high molecular weight colloids (dextrans, oxypolygelatin, and hetastarch) may have additional effects on hemostasis, such as causing greater decrements in plasma [Fbg] and VWF:Ag. The effects depend on the fluid infused, the rate of infusion, and the recipient species.[364]

THROMBOSIS

I. *Thrombosis* is the formation of thrombi, which are in vivo "clots" adherent to the vessel or heart wall and composed of varying amounts of fibrin and blood cells. Thrombi that form under high-flow conditions (arterial) contain more platelets (white thrombi). Thrombi that form under low-flow conditions (venous) contain more RBCs and fewer platelets (red thrombi).

A. Thrombi may cause clinical signs by obstructing blood flow and causing ischemia, by forming emboli (fragments) that travel to distant sites and cause ischemia, or by consuming platelets and other hemostatic factors, which may lead to hemorrhage.

B. They may occur at a single site because of a local disturbance (e.g., chemical irritants injected into the jugular vein) or at multiple sites because of more systemic abnormalities (DIC).

C. Clinical signs vary with location and severity. Dyspnea may occur with pulmonary thromboembolism, hematuria with renal infarction, edema of the head with cranial vena cava thrombosis, and ascites with portal vein thrombosis. Distal aortic thrombosis may cause rear-limb weakness and pain, decreased femoral pulse pressure and quality, and cool extremities.

II. Prothrombotic and thrombotic states: A prothrombotic state is one in which the balance between prothrombotic factors and antithrombotic factors shifts to favor thrombosis (i.e., a state

of *thrombophilia*); there may be subclinical consumption of hemostatic factors without clinical thrombosis, but thrombosis occurs if the balance shifts enough.

A. Predisposing factors are usually thought of broadly in terms of alterations in (i) blood flow, (ii) coagulation, or (iii) blood vessels (including endothelial cells, which play huge prothrombotic and antithrombotic roles).
   1. Predisposing abnormalities of blood flow include blood stasis (typically venous) and turbulent blood flow (typically arterial), which can relate to vascular anomalies, cardiomyopathy, or hyperviscosity.
   2. Predisposing factors for increased coagulation include platelet activation (via vascular damage, turbulence, or inflammatory mediators), increased cytokine- or endotoxin-induced intravascular TF expression, neutrophil extracellular traps, decreased endogenous anticoagulants, and impaired fibrinolysis.
   3. Blood vessels become more prothrombotic when damaged or stimulated by inflammatory mediators or thrombin. Inflammation is a common underlying state.

B. The risk of thrombosis (localized or disseminated) increases with more predisposing factors (a more prothrombotic state). Acquired conditions associated with thrombosis include the following.[204,205,365]
   1. Cardiovascular disease: vasculitis (e.g., heartworm disease, *Strongylus vulgaris* larvae in horses), endocarditis, intravascular catheterization, congestive heart failure, feline cardiomyopathy, atrial fibrillation, injection of chemical irritants, vascular defects, extracorporeal circuits, and cardiac pacing leads
   2. Severe inflammatory disease: sepsis, endotoxemia, equine colic and colitis, and systemic inflammatory response syndrome (SIRS); the prothrombotic states of canine immune-mediated hemolytic anemia and pancreatitis may relate to inflammation and other factors
   3. Severe surgical or nonsurgical tissue trauma: includes necrosis, burns, heatstroke
   4. Malignant neoplasms: may involve disorders of vessels, inflammation, tissue damage, and other processes
   5. Protein loss or anticoagulant-deficient states (AT, protein C): protein-losing nephropathies and enteropathies in dogs, hepatic disease
   6. Hepatic disease: hepatic abscesses in cattle and horses; various hepatic disorders in dogs, particularly with other predisposing states (causing portal vein and/or splenic vein thrombosis)[366]
   7. Endocrine disorders: hypothyroidism, canine hyperadrenocorticism
   8. Glucocorticoids: Use of glucocorticoids has been associated with thrombosis in dogs and may be a predisposing factor, but many of the underlying conditions for which glucocorticoids were administered are themselves prothrombotic states which makes the roles of glucocorticoids unclear.
   9. Antiphospholipid antibodies (rare): promote thrombosis in vivo but may prolong coagulation times in vitro

III. Hemostasis testing for thrombotic disease is very limited, especially for early recognition. Some disorders are known to be prothrombotic, so relevant clinical and laboratory diagnostics can be monitored, and a few disorders are so consistently prothrombotic that prophylactic antithrombotic therapy is used routinely once a diagnosis is reached.[367] Imaging studies may detect large thrombi but is a relatively insensitive approach. The following constellation of hemostasis tests is also limited but may be used to assess for prothrombotic and thrombotic states. The tests are most useful as a panel interpreted in conjunction with clinical status and

other appropriate tests such as diagnostic imaging and blood gas analysis. Even when used together, these tests neither reliably predict nor reliably exclude clinically relevant thrombi. Assessment of thrombotic disease is further complicated by the rapid postmortem fibrinolysis of thrombi, making some undetectable.

A. Increased [D-dimer] is evidence of cross-linked fibrin formation and degradation and therefore intravascular coagulation. Increased [FDP] provide similar support but could occur with fibrinogenolysis. However, increases in each occur with other conditions such as inflammatory disease or internal hemorrhage in the absence of clinically relevant thrombi.
B. Decreased AT activity can be evidence of consumption caused by intravascular coagulation, but it may be the result of AT loss or decreased production that is promoting thrombus formation.
C. Decreased protein C activity can be evidence of consumption caused by intravascular coagulation, but it may also result from other causes, such as vitamin K antagonism or hereditary deficiency, in which case it may contribute to intravascular coagulation.
D. Thrombocytopenia can be evidence of consumption caused by intravascular coagulation, but there are many other causes (see Chapter 4).
E. Decreased [Fbg] can be evidence of consumption caused by intravascular coagulation, but increased production associated with inflammation may mask shortened Fbg survival, and hyperfibrinogenemia has been associated with thrombosis in a variety of disorders.
F. Increased [TAT] is evidence of increased thrombin generation and therefore intravascular coagulation, but the test is not widely available.
G. VET may provide support for a prothrombotic state but is discussed in a separate section.
H. Other analytical approaches are under investigation or require species-specific antibodies that are not available. Measurement of the kinetics and degree of thrombin generation in a plasma sample, assessing for prothrombotic microparticles by flow cytometry, and assaying for neutrophil extracellular traps or free deoxyribonucleic acid (DNA) are under investigation. Antibodies are not available for measuring prothrombin fragments that form when FII is cleaved to form FIIa, or for fibrinopeptides that form when thrombin acts on Fbg in domestic mammals.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 6

BONE MARROW AND LYMPH NODES

Michael A. Scott and Steven L. Stockham

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Table 6.1. Abbreviations and symbols in this chapter	
ALSG	Animal Leukemia Study Group
AML	Acute myeloid leukemia
CBC	Complete blood count
CD	Cluster of differentiation or designation
DNA	Deoxyribonucleic acid
Epo	Erythropoietin
Fe	Iron
FeLV	Feline leukemia virus
G:E	Granulocytic to erythroid
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
IMHA	Immune-mediated hemolytic anemia
M:E	Myeloid to erythroid
M6-Er	Acute erythroleukemia with erythroid predominance
MDS	Myelodysplastic syndrome
MDS-EB	Myelodysplastic syndrome with excess blasts
MDS-Er	Myelodysplastic syndrome with erythroid predominance
MDS-RC	Myelodysplastic syndrome with refractory cytopenia
MPD	Myeloproliferative disease
MPO	Myeloperoxidase
NK	Natural killer
nRBC	Nucleated red blood cell (all erythroid precursors)
PARR	Polymerase chain reaction for antigen receptor rearrangements
PCR	Polymerase chain reaction
PIMA	Precursor-targeted immune-mediated anemia
PRCA	Pure red cell aplasia
RBC	Red blood cell (erythrocyte)
TCRαβ	αβ-Antigen–binding heterodimer of the T-lymphocyte receptor (T-cell receptor)
TCRγδ	γδ-Antigen–binding heterodimer of the T-lymphocyte receptor (T-cell receptor)
WBC	White blood cell (leukocyte)
WHO	World Health Organization

BONE MARROW: MAJOR CONCEPTS AND TERMS

I. Composition of bone marrow

A. *Bone marrow* is the tissue enclosed by cortical and cancellous bone, and its major function is *hematopoiesis*, the production of hemic cells found in blood and other tissues. Bone marrow consists mostly of *hematopoietic cells* (stem cells and their developing progeny), adipose tissue, and supportive tissue. When hematopoietic cells predominate, as in young animals, the marrow appears red. When adipose tissue predominates, as in the diaphyseal regions of long bones in healthy adults, the marrow appears yellow because of its high fat content. The extent of red marrow (active hematopoiesis) diminishes as neonates grow to adult size, but red marrow remains within bones of the axial (ilium, ribs, sternebrae, and vertebrae) and

proximal appendicular (humeri and femurs) skeleton. Yellow marrow can convert to red marrow during pathologic states that stimulate hematopoiesis and return to yellow marrow with resolution of the pathologic state.

B. Nutrients are delivered by vessels branching from one or more nutrient (medullary) arteries that enter through the cortical bone. New hemic cells enter blood through the walls of medullary sinuses.

C. Hematopoietic cells develop in a supportive extravascular microenvironment controlled by myriad local and systemic cytokines.

II. Hematopoietic cells: Microscopically recognizable hematopoietic cells are in the proliferation pools and maturation pools of the megakaryocyte, RBC, and WBC lineages. These are *precursors* of hemic cells that arise from *progenitor cells*, stem cells that cannot be detected by routine bone marrow evaluation.

A. Megakaryocyte lineage

1. *Megakaryocytes* – from the Greek *megas* (large) + *karyon* (nucleus) + *kytos* (cell) – are very large polyploid cells, each with the capability of producing many platelets.
2. Thrombopoietin and other cytokines (see Chapter 4) stimulate the formation of mature megakaryocytes from megakaryoblasts. This occurs by *endomitosis*: a cell's nucleus divides repeatedly without cytokinesis, thus forming cells of progressively greater ploidy (most ~16N but can be much greater).
   a. *Megakaryoblasts* are similar in size and appearance to rubriblasts, myeloblasts, and monoblasts, having single nuclei and deeply basophilic cytoplasm; cell contours may reflect cytoplasmic blebbing. Some sources include later cell stages as megakaryoblasts.
   b. *Promegakaryocytes* develop from megakaryoblasts and are intermediate stages between megakaryoblasts and megakaryocytes. Some define them as having what appear to be two or four distinct nuclei and scant amounts of basophilic cytoplasm that may form blebs, while others also include later basophilic megakaryocytes with greater cytoplasmic granulation, ploidy, and nuclear lobulation (Figure 6.1).
   c. Megakaryocytes vary in size and ploidy. As they develop from promegakaryocytes, they become larger, have more nuclear lobulations, and have more abundant, paler cytoplasm that extends into sinusoids as long pseudopods that break up into anucleate platelets. Mature forms predominate (Figure 6.1).

B. RBC lineage

1. In veterinary medicine, the nucleated erythroid precursors are usually named using a *rubri-* prefix (Table 6.2). Development occurs along a continuum, making consistent cell subclassification problematic. Metarubricytes, the last stage, give rise to reticulocytes which become mature RBCs.
2. As cells proliferate and mature (Figure 3.1) from the first recognizable stage, the rubriblast, they decrease in size and cytoplasmic basophilia while increasing hemoglobin content and associated eosinophilic staining (Figure 6.2A and C).
3. In human medicine, and sometimes in veterinary medicine, the nucleated erythroid precursors are named by using either erythroblast or normoblast terms (Table 6.2).
4. Accordingly, an increased concentration of nucleated erythroid precursors in the blood may be called *rubricytosis* in veterinary medicine, but it is also called either *erythroblastosis* or *normoblastosis* as in human medicine, and it may be referred to simply as *increased nRBCs*. The term rubricytosis does not refer only to the rubricyte stage; it refers to all stages, each of which contains *rubri* in the name. The term *metarubricytosis* is also used, but rubricytes commonly accompany metarubricytes, so this term may be imprecise.

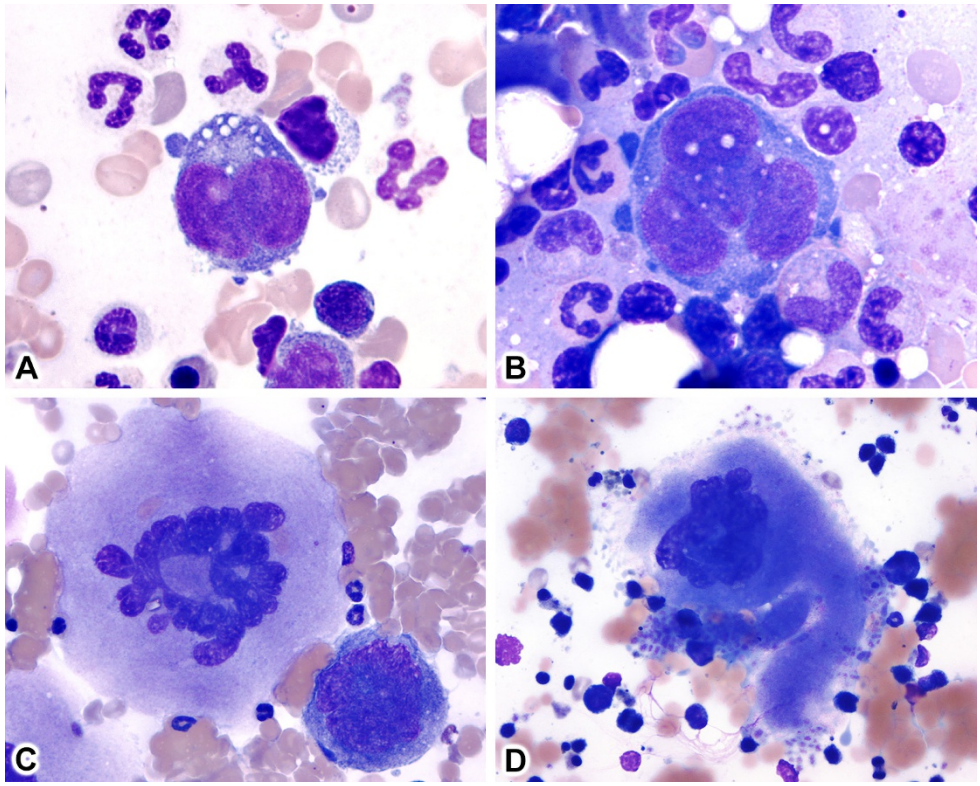


Figure 6.1. A. Promegakaryocyte (4N ploidy) with cytoplasmic blebs. B. Immature megakaryocyte with relatively scant basophilic cytoplasm. C. Mature megakaryocyte (high ploidy with abundant cytoplasm) and smaller immature megakaryocyte, half the magnification of A and B. D. Megakaryocyte with a trunk-like pseudopod, same magnification as C. Canine bone marrow aspirates.

Table 6.2. Nomenclature of the erythroid series

Rubricyte system[a]	Erythroblast system[b]	Normoblast system[b]
Rubriblast	Proerythroblast	Pronormoblast
Prorubricyte	Basophilic proerythroblast	Basophilic normoblast
Basophilic rubricyte	Basophilic erythroblast	Basophilic normoblast
Polychromatophilic rubricyte	Polychromatophilic erythroblast	Polychromatophilic normoblast
Normochromic rubricyte	Early orthochromatic erythroblast	Early orthochromatic normoblast
Metarubricyte	Late orthochromatic erythroblast	Late orthochromatic normoblast

[a] Usual nomenclature in veterinary medicine
[b] Nomenclature used primarily in human medicine

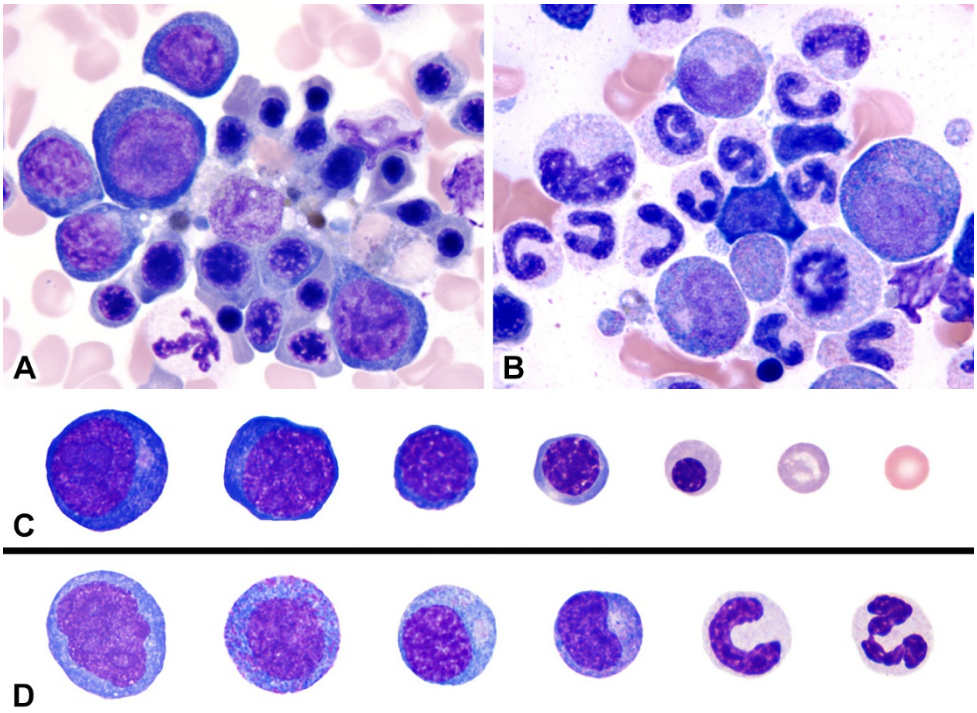


Figure 6.2. A. Erythropoiesis from rubriblasts through metarubricytes around a nurse macrophage in a dog with erythroid hyperplasia and a decreased M:E ratio. B. Neutropoiesis from a myeloblast through mostly band neutrophils, including a giant band neutrophil, from a dog with immune-mediated neutropenia, myeloid hyperplasia, and an increased M:E ratio. C. Progression of erythropoiesis: rubriblast, prorubricyte, early and later rubricytes, metarubricyte, polychromatophil, mature RBC, dog. D. Progression of neutropoiesis: myeloblast, progranulocyte (promyelocyte), myelocyte, metamyelocyte, band neutrophil, segmented neutrophil, dog.

C. Nonlymphoid WBC (myeloid) lineages
   1. Nonlymphoid WBC precursors are often collectively labeled myeloid cells. However, there are different meanings of *myeloid* and the prefix *myelo-*. Thus, a word beginning with *myelo-* must be interpreted in context.
      a. *Myel-* or *myelo-* meanings:
         (1) Combining form denoting the relationship to bone marrow
         (2) Referring to the nonlymphoid hemic cells of the megakaryocyte, WBC, and erythroid series (as in myeloproliferative neoplasms)
         (3) Referring to granulocytes, as in myelomonocytic leukemia, which is a neoplasm with granulocytic (myelo) and monocytic origins
         (4) Often used in specific reference to spinal cord
      b. *Myeloid* meanings:
         (1) Pertaining to or derived from bone marrow
         (2) Pertaining to nonlymphoid hemic cells of the megakaryocyte, granulocyte, monocyte, and erythroid series, as in acute myeloid leukemia (which includes pure erythroid leukemia)

(3) Pertaining to all nonlymphoid WBCs (granulocyte, monocytes) and their precursors, as in the myeloid to erythroid ratio
(4) Having the appearance of the myelocyte
(5) Pertaining to spinal cord

c. *Myelocyte* meanings:
(1) Intermediate precursor cell of the granulocyte series
(2) Any cell of the grey matter of the nervous system

d. *Myelitis* meanings:
(1) Inflammation of bone marrow
(2) Inflammation of the spinal cord

e. *Myelogenous* meanings:
(1) Produced in bone marrow
(2) Pertaining to the nonlymphoid hemic cells – megakaryocyte, WBC, and erythroid lineages – as in acute myelogenous (myeloid) leukemia

2. Nonlymphoid WBCs: monocytes, granulocytes (neutrophils, eosinophils, and basophils), mast cells, and dendritic cells (see Chapter 2 and Figure 2.1)
a. Neutrophils predominate. The mature neutrophils in the bone marrow are collectively referred to as the *neutrophil storage pool*.
b. Granulocytes are derived from myeloblasts and promyelocytes, also called progranulocytes, a term that emphasizes they do not form monocytes. Cells become smaller and develop specific secondary granules; acquisition of secondary granules is usually described as occurring at the myelocyte stage (neutrophil myelocyte, eosinophil myelocyte, and basophil myelocyte), but secondary granules (e.g., eosinophil granules) can be present in earlier stages. Further maturation includes nuclear indentation and segmentation (Figure 6.2B and D).
c. Monocytes develop from monoblasts and promonocytes, populations that are inconspicuous and difficult to identify with certainty in bone marrow from healthy animals. Promonocytes lack the cytoplasmic granulation that typifies promyelocytes (progranulocytes). Macrophages, derived from monocytes, are present in low numbers.
d. Mast cell precursors are not detectable microscopically. Few resident mast cells derived from hemic stem cells may be present.

D. Lymphoid lineage (lymphocytes and plasma cells)
1. Lymphocytes and plasma cells are present in relatively low numbers in health.
2. All lymphoid cells are derived from a common bone marrow precursor cell (Figure 2.1).
3. Cells destined to be B lymphocytes mature in the bone marrow or certain other tissues, whereas cells destined to be T lymphocytes migrate to the thymus and mature there. Also, many lymphocytes form by mitosis of stimulated mature lymphocytes.
4. Plasma cells develop from B lymphocytes and have a large capacity to produce immunoglobulins.

E. Dendritic cells
1. Dendritic cells are specialized for uptake, transport, processing, and presenting antigens to T lymphocytes. There are several subtypes, including antigen-presenting conventional dendritic cells, plasmacytoid dendritic cells, and monocyte-derived dendritic cells.
2. All dendritic cells are derived from a common progenitor, but monocyte-derived dendritic cells differentiate along a different path. Differentiation to dendritic cells is not detectable through microscopic evaluation of bone marrow.
3. They are a rare and unrecognized cell type in blood smears, most being immature and lacking the conventional dendritic appearance they acquire after leaving the blood. Some monocytes that extravasate to tissue may form dendritic or dendritic-like cells.

F. Bone marrow nucleated cell differential count in health
1. The expected ratio of nonlymphoid WBCs to erythroid precursors (M:E ratio; see Section V. Bone marrow evaluation) varies with the species and the individual. Average reported ratios vary from just under 1.0 in large animals, reflecting more erythroid than myeloid cells, to 1.6 in cats, reflecting more myeloid than erythroid cells (see M:E ratio in Section V.B.2.b).
2. In health, cells of the neutrophil and erythroid series predominate, and the majority of cells in each lineage are the more-mature cells.[1]
   a. Myeloblasts and promyelocytes account for < 4 % of the nucleated cells.
   b. Segmented and band neutrophils are the predominant WBCs.
   c. Cells of the eosinophil series usually account for < 5 % of the nucleated cells. Cells of the basophil series are rare.
   d. Rubriblasts and prorubricytes account for < 4–5 % of the nucleated cells.
   e. Metarubricytes and late-stage rubricytes are the predominant nucleated RBCs. Polychromatophilic RBCs are present because they are not released to blood immediately when formed, but they do not form a storage pool.
3. Lymphocytes usually account for < 5 % of the nucleated cells, except in cats.
   a. Up to ~20 % lymphocytes have been reported in healthy cats, and similar percentages have been reported in some healthy adult dogs.[2]
   b. Plasma cell percentages have been as high as 2–3 % in some canine studies.
4. Cells of the monocyte series constitute < 1–2 % of nucleated cells, but their identification may be unreliable. Recognizable macrophages constitute < 1 % of the cells in health.
5. Developing and mature megakaryocytes account for < 0.5 % of hematopoietic cells in health.[2]

III. Indications for bone marrow examination
A. Unexplained and persistent (> 5 d) cytopenia (most common indication)
1. Nonregenerative anemia: Include a core to assess for myelofibrosis if the RBC population is macrocytic and hypochromic with ovalocytosis.
2. Neutropenia
3. Thrombocytopenia: Marrow findings that alter clinical management are uncommon for dogs with selective thrombocytopenia, particularly when thrombocytopenia is marked.[3] Aspirate smears must contain marrow particles to adequately assess the degree of megakaryopoiesis.
4. Bicytopenia or pancytopenia: A core biopsy may be particularly helpful to better assess hypoplasia.
B. Evidence of an occult bone marrow neoplasm
1. Unexplained atypical cells (blasts, dysplastic cells) in blood
2. Unexplained hyperproteinemia and hyperglobulinemia (potential plasma cell myeloma)
3. Unexplained free hypercalcemia (potential hypercalcemia of malignancy)
C. Unexplained and persistently increased cell concentrations (less common)
1. Lymphocytosis
2. Thrombocytosis, especially extreme
3. Erythrocytosis, for suspected polycythemia vera
4. Granulocytosis or monocytosis if chronic leukemia is suspected
5. Mastocytemia
D. To search for the following:
1. A metastatic neoplasm, especially of lymphocytes and mast cells

2. Possibly Fe stores, especially when considering Fe deficiency, but CBC findings are usually diagnostic; healthy cats and young mammals lack marrow hemosiderin
3. Evidence of certain specific diseases: histoplasmosis, leishmaniasis, or lysosomal storage diseases (commonly diagnosed without bone marrow evaluation)
4. An explanation for a fever of unknown cause

IV. Methods of bone marrow collection and processing

A. Complete descriptions of a bone marrow biopsy (collection, fixation, staining, and cytologic or histologic examination of bone marrow from a living animal) are beyond the scope of this text. Procedures of a bone marrow biopsy are described in several sources.[1,4–13] Based on clinical experience and published information, adverse effects of bone marrow collection are usually limited to pain, mild lameness, or bruising;[14] fractures are extremely rare. As with any invasive procedure involving anesthesia, there is a small potential for infections or anesthetic complications. Thrombocytopenia is not a contraindication given that (i) external hemorrhage can be controlled and (ii) intramedullary administration of blood is an emergency approach to transfusion.

B. Typically, bone marrow samples are collected from sites that are expected to have active hematopoietic tissue (red marrow).
   1. In dogs and cats, such tissue is expected in the iliac crest and the medullary cavities of proximal femurs or humeri, sternum, and ribs. The ilium and proximal humerus are common sites for aspirate and core biopsies, but the ilium may be too narrow to sample in cats and small dogs. Active hematopoiesis may become less prevalent in the proximal appendicular skeleton with age.
   2. In horses, active bone marrow is most accessible in sternebrae (third or fourth); the sternum has also been recommended for ruminants, llamas, and alpacas. In cattle, medullary cavities of dorsal ribs can be sampled.

C. The two major methods of collecting antemortem bone marrow biopsy samples are aspiration and core cutting.
   1. *Aspiration*: A Rosenthal or Illinois biopsy needle is inserted through cortical bone and into the marrow of an appropriate site, either manually or with a powered rotary device.[15] The stylet is removed and bone marrow is aspirated.
      a. Aspiration may be into a syringe containing EDTA anticoagulant, in which case the material is expelled into a Petri dish and marrow particles are collected by pipette to make particle-rich smears. Material remaining after smears are made can be saved and used for flow cytometric evaluation or PCR testing for infectious agents or clonality. Aspirated material can also be processed to form cell blocks of fixed cells for histologic evaluation, but this is not commonly done.
      b. When no anticoagulant is used, excessive hemodilution is controlled by stopping marrow aspiration as soon as blood is seen entering the syringe. Material is immediately expelled, and smears are promptly made, preferably from particle-rich portions of the sample.
      c. The Rosenthal and Illinois needles are straight shafted with a beveled end. Nondisposable forms require sterilization and sharpening. Disposable forms are available.
      d. Routine hypodermic needles (1 inch, 20 gauge) were effective for collecting sternal marrow aspirate samples from beagles.[16]
   2. *Core cutting*: A Jamshidi biopsy needle is inserted into the cortical bone over an appropriate bone marrow collection site unaffected by any preceding aspirate. The stylet is removed, the needle is advanced into the bone marrow with a forceful twisting

motion, the needle is rotated 360° several times, and then the needle is withdrawn. After the needle is withdrawn, the stylet is used to push the core sample out the top of the needle (not the tip end that is narrower than most of a Jamshidi needle's bore); this promotes retrieval of a cylindrical core of tissue. Other intraosseous needles and powered rotary devices may alternatively be used.[15,17,18]

3. Combining examinations of core and aspirate marrow samples provides the most complete information because each provides information the other cannot, and it is often not known prior to assessment if the added value of a core will be important in a particular case. Also, collecting both sample types increases the chances of obtaining a sample of diagnostic quality; about 30 % of submitted aspirate samples were considered to be of nondiagnostic quality in one study.[19] However, collecting both samples increases the cost and risk to the patient, and the core may not be needed for a diagnosis.
   a. Cytologic evaluation of aspirates is almost always indicated.
   b. Histologic examination is useful when marrow architecture is required to reach a diagnosis, myelofibrosis is suspected (connective tissue exfoliates poorly), or hypoplastic states are suspected (e.g., when bicytopenia or pancytopenia is present), in which case an aspirate may yield few cells and a pattern that may not be distinct from poor sampling.
   c. When both samples are collected, care should be taken to avoid diminishing the quality of the second sample by the first collection procedure. A core can be artifactually altered when collected after an aspirate in the same area, and an aspirate may be more hemodiluted if collected after a core sample at the same site. This should be avoided by (i) collection from different bones or (ii) collection from the same bone and skin incision but through a separate marrow entry point and path, i.e., directing the needle so that the core path does not cross the aspiration track.
   d. Combined aspirate and core collection methods with a single needle penetration have been described whereby the core is collected immediately after the aspirate by advancing the needle beyond the point of aspiration.[15,20] In postmortem comparisons, cores collected in this manner were shorter and had more hemorrhage than paired control cores collected directly without first aspirating.[20] Diagnostic value appeared adequate, but more hemorrhage may occur with antemortem collection. A similar combined method yielded what were considered to be samples of diagnostic quality from clinical patients with hematologic disorders.[15]

D. Processing bone marrow aspirate samples
   1. If the needle and syringes are just wetted with anticoagulant or if no anticoagulant is used, it is critical that samples be distributed onto glass slides within seconds of collection so as to reduce clot formation and in vitro changes. A variety of smear, "squash", roll, and imprint methods may be used to produce slides that have optimal zones of intact cells and foci of bone marrow particles, both of which are essential for complete interpretation.
      a. After aspiration, drops may be placed on a series of glass slides and quickly spread by either a wedge blood smear technique or a horizontal "squash" technique. The latter better spreads cells that are present within particles. However, drops may be large, resulting in smears that are too long or too thick. To overcome this, one can lift particle-rich parts of each drop up with the underside of the short edge of a spreader slide and then use that spreader slide to make squash preparations with these smaller amounts of sample. Another option is to express the marrow at the top of inclined slides so that the blood flows downward onto absorbent paper, leaving a more particle-rich sample behind for smearing.

b. Alternatively, collected material can be placed in a watch glass or Petri dish with anticoagulant, and the particles can be extracted and spread onto slides with less concern about clotting. Prompt smearing is still recommended.

2. Aspiration may yield a "dry tap" containing essentially no material, in which case additional aspiration attempts or core biopsy samples are required. "Dry taps" do not indicate that the marrow is hypocellular; they may occur with bone marrow hypercellularity in a variety of disorders.
3. For routine microscopic examinations, the air-dried samples are stained with a stain that is used for blood films (a Romanowsky stain such as a Wright stain or Diff-Quik). A Prussian blue stain may be applied to assess the amount of stainable Fe; this also allows assessment of Fe inclusions in erythroid cells.
4. Processing requirements for cytochemical, immunocytochemical, and PCR procedures should be obtained from the relevant laboratory.

E. Processing core biopsy samples

1. After making imprints by touching or rolling the core on a glass slide, the core sample is placed in a fixative. B5, Zenker's fixative, or acetic acid-zinc-formalin provide better cell detail than routine buffered formalin, but B5 and Zenker's fixatives have the disadvantage of containing mercury, which must be handled and disposed of appropriately. Fixation procedures vary with the type of fixative that is used. The fixed core sample is submitted to a hematology or histology laboratory that can process and stain sections of the sample for microscopic examination.
2. Air-dried smears should be kept away from fixatives because formalin fumes can severely alter the staining properties of cells. If mailing samples to a lab, place the unstained slides and fixed tissue container in separate doubled zip-sealed bags.
3. Core samples require decalcification before the sample can be sectioned and prepared for staining. The decalcification method must be mild, not the routine method used for bony samples; such methods cause too much cell damage to allow differentiation of cell types. After sectioning, a variety of stains and immunohistochemical labels may be applied to demonstrate the types of cells or other contents of the sample.

V. Bone marrow examination

A. Gross examination

1. Aspirated bone marrow samples contain peripheral blood cells, but the best samples have little contaminating blood. A representative sample typically contains small pieces of tissue known as bone marrow particles, fragments, spicules, units, or grains. Those containing adipose tissue glisten.
2. The appearance of bone marrow core samples varies. They may be hypercellular and red, hypocellular and white (fatty), or consist of only cortical bone or blood clots.

B. Microscopic examination: A complete microscopic examination of bone marrow samples involves the characterization of the bone marrow tissue architecture, differentiation and enumeration of cells, and evaluation of individual cell structure. Such examinations are best completed on quality stained histologic and cytologic samples with a quality microscope by people trained for such examinations, i.e., board-certified veterinary pathologists with extensive knowledge of hematologic disorders. The following bone marrow features are routinely assessed:

1. Cellularity or hematopoietic cellularity
   a. *Cellularity* generally refers to the proportion of marrow that is filled with hematopoietic cells rather than adipocytes, being about 25–75 % in healthy adults, the 75 % being more reflective of young adults and the 25 % more reflective of old

adults.[21] The density of the cells in these areas also reflects cellularity. Although cellularity has been described as the proportion of cells to fat, the fat is in adipocytes (cells), so it is actually the proportion of space filled by nonadipocytes to that filled by adipocytes. It is assessed at low magnification, in aspirate particles or tissue sections; space filled by supportive stroma and vessels is typically disregarded. Increases in cellularity may be from (i) increased hematopoietic cells responding to disease, (ii) inflammatory infiltrates, or (iii) neoplastic cells, primary or metastatic.

b. *Hematopoietic cellularity* is similar to cellularity but specifically refers to the proportion of marrow space filled by cells engaged in hematopoiesis; this would exclude inflammatory infiltrates and neoplastic populations not contributing to the production of functional blood cells. For example, when marrow contains many neoplastic plasma cells because of a plasma cell myeloma, it may be of high cellularity, but the neoplasm may have replaced most of the active hematopoietic tissue, thus yielding low hematopoietic cellularity and corresponding cytopenias. In such cases, some hematopathologists report the adipocyte cellularity (percentage of space filled by adipocytes) rather than the hematopoietic cellularity; a low adipocyte cellularity implies high marrow cellularity but does not indicate high hematopoietic cellularity.

c. Marrow cellularity can vary substantially among medullary spaces. When this occurs, the range and mean cellularity may be reported.

d. If few particles are present in cytologic preparations, any assessment of cellularity is likely unreliable. The degree of hematopoiesis cannot be determined cytologically in the absence of marrow particles.

e. Cellularity is best assessed in core samples (if they were collected from appropriate anatomic sites). Cores can be particularly helpful when aspirate particles contain only fat that may be from extramedullary adipose tissue rather than bone marrow. Also, because particles can be thick, cellularity may be overestimated from the cumulative effect of hemic cell clusters at different depths throughout the particles, a problem not encountered with thin histologic tissue sections. Overestimating cellularity can substantially alter interpretation. Concordance in clinical pathologists' estimates of particle hematopoietic cellularity can be relatively modest, even with just two individuals evaluating samples from healthy dogs.[22] Pathologists who have assessed numerous paired aspirate and core samples, not just aspirate smears, are likely best prepared to provide reliable estimates of particle hematopoietic cellularity.

f. If bone marrow particles or medullary spaces of core sections contain > 75 % adipose tissue on average, the bone marrow is hypocellular (Figure 6.3A). If bone marrow particles or medullary spaces contain < 25 % adipose tissue overall, the bone marrow is hypercellular (Figure 6.3B).

g. Cellularity of an aspirate smear (not its particles) does not necessarily reflect hematopoietic cellularity of the bone marrow. Good aspirate smears of moderate or high cellularity may contain particles with overall decreased hematopoietic cellularity. If bone marrow particles are not present and the preparation is very cellular, the bone marrow is likely hypercellular and unlikely hypocellular. If bone marrow particles are not present and the preparation is not very cellular, the bone marrow may be hypocellular, or the sample may be poorly representative of bone marrow tissue.

2. Hematopoietic populations (Figures 2.1 and 6.4)

a. Number of megakaryocytes

(1) The number of megakaryocytes per average marrow particle (cytology) or medullary space (histology) is subjectively assessed. Assessment is not reliable in aspirate smears with few particles, because megakaryocytes tend to exfoliate with

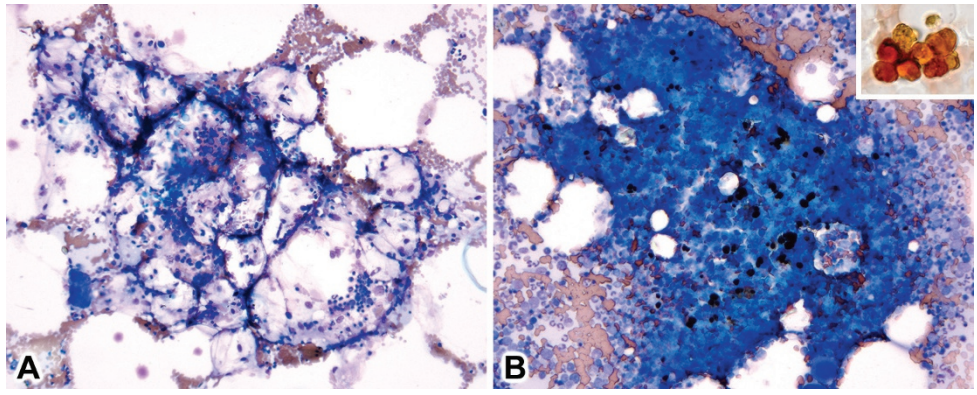


Figure 6.3. A. Hypocellular bone marrow particle consisting mostly of adipocytes and associated with lipid spaces around the particle, dog with generalized marrow hypoplasia. B. Hypercellular particle containing little fat and prominent dark-staining hemosiderin in macrophages, dog with myeloid hyperplasia and immune-mediated neutropenia (same dog as Figure 6.2B). Insert shows the appearance of unstained hemosiderin globules.

particles. Although histologic samples generally provide a more reliable assessment of megakaryocyte numbers, early megakaryocyte stages are difficult or impossible to detect with routine histologic evaluation.

(2) In good samples, finding more megakaryocytes than expected in health supports megakaryocytic hyperplasia or, rarely, neoplasia. Fewer megakaryocytes than expected in health supports megakaryocytic hypoplasia.

b. M:E ratio

(1) A M:E ratio may be estimated or calculated by differentiating 500–1000 consecutive myeloid and nucleated erythroid precursors in cytologic samples and then dividing the number of myeloid cells by the number of erythroid cells. For example, if out of 500 counted cells there were 300 myeloid cells and 200 nRBCs, the M:E ratio would be 300/200 = 1.5. Lymphocytes, plasma cells, macrophages, mast cells, and stromal cells are not included as myeloid cells; neutrophils, eosinophils, basophils, monocytes, and their precursors are included.[23] Megakaryocytes are usually not included, but they generally make little difference because there are few of them compared to other cell types.

(a) Some authors refer to G:E ratios rather than M:E ratios and may or may not use G:E and M:E as synonyms. Technically, a G:E ratio excludes cells of the monocyte lineage, but it is difficult to definitively differentiate monoblasts and promonocytes from granulocyte precursors. The difference between these ratios is usually insignificant because there are usually few cells of the monocyte series.

(b) The accuracy of quantitative cytologic M:E ratios is impacted by several factors including (i) variations in M:E ratios from one area of a slide (and of bone marrow) to another or from one slide to another, (ii) the percentage of lysed cells that are present and therefore not included in counts, (iii) the relatively low number (e.g., 500) of cells counted to represent all the cell types in the marrow, and (iv) the quality (e.g., thickness, staining) of areas assessed.

(2) Reported mean M:E ratios determined cytologically for healthy dogs, cats, horses, cattle, and llamas vary from about 0.3 to 3.0, most being near 1.0–2.0.

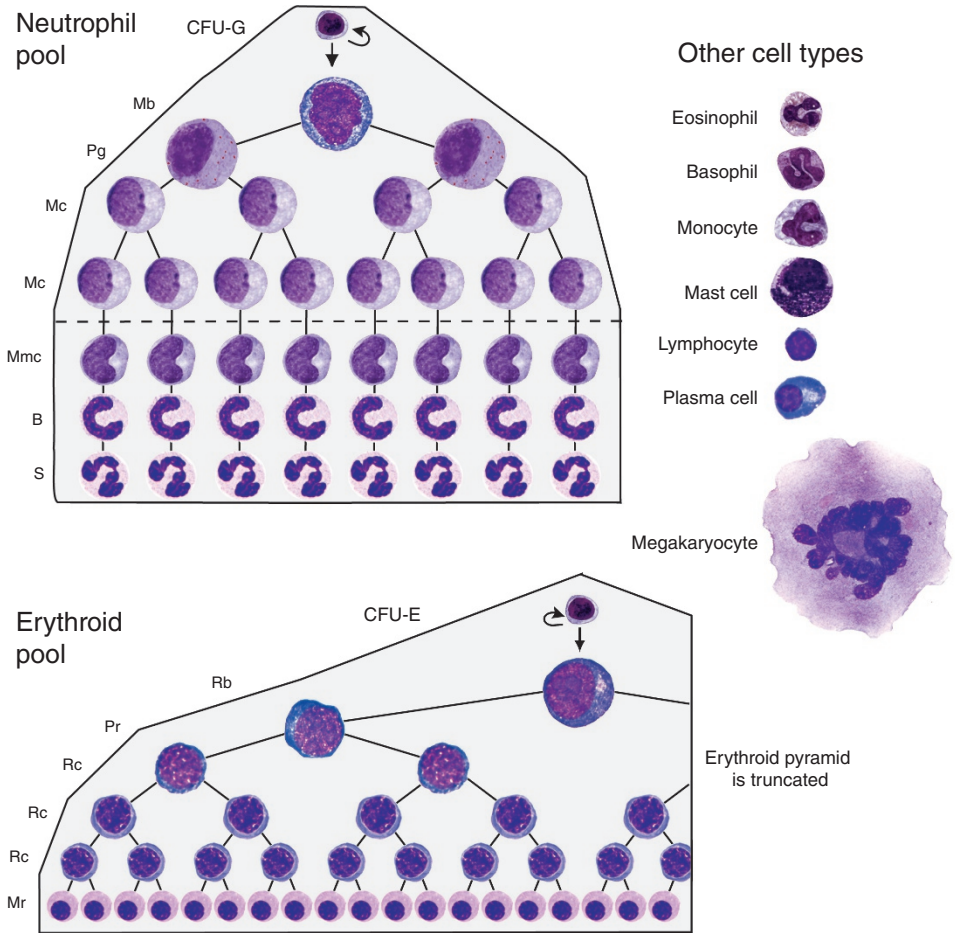


Figure 6.4. Hemic cells found in the bone marrow of healthy mammals. The two major pools are cells of the neutrophil series in the myeloid pool and cells of the erythroid pool. Eosinophils, basophils, and their precursors are also components of the granulocytic pool within the myeloid pool, but they are present in low numbers in health. Monocytes are part of the myeloid pool but not the granulocytic pool; their numbers are typically low and have little impact on the M:E ratio. Macrophages are differentiated cells that are not included in the myeloid pool when evaluating bone marrow hematopoietic activity. Like macrophages, mast cells are terminally differentiated tissue cells rather than hematopoietic cells and are not included in the M:E ratio. Lymphocytes and plasma cells belong to the lymphoid pool, not the myeloid pool. Megakaryocytes may be considered part of the myeloid pool but are often evaluated as a separate cell line and not included in the M:E ratio, as is the case here.
B, band neutrophil; CFU-E, colony-forming unit–erythroid; CFU-G, colony-forming unit–granulocyte; Mb, myeloblast; Mc, myelocyte; Mmc, metamyelocyte; Mr, metarubricyte; Pg, progranulocyte; Pr, prorubricyte; Rb, rubriblast; Rc, rubricyte; and S, segmented neutrophil

(a) Dogs have been assessed most. Their mean M:E ratio is about 1.1–1.2,[1,2,6,24,25] but a mean G:E ratio of 0.9 has been reported.[22] Variation within healthy dogs is quite large, with values as low as about 0.5 and as high as ~3 in dogs within a single study.[2]

(b) Data are less available for other species.[6,13] The mean M:E ratio of cats tends to run a little greater than for dogs, that of horses a little lower, and that of cattle about 0.7–0.8.[26] The mean was 1.9 in a small group of llamas.[27]

(c) Differences in M:E ratios among collection sites have been suggested but do not clearly exist. In one study, mean values in healthy dogs were mildly greater in the ilium compared to the humerus (1.3 versus 1.1),[25] but, in another study, the iliac M:E ratio had a mean of about 1.1.[2]

(3) For the experienced hematopathologist, subjective estimates of the M:E ratio combined with the overall pattern of blood and bone marrow findings are usually sufficient for diagnostic purposes.

(4) With routine histologic samples, the M:E ratio is subjectively estimated, but estimates may be unreliable with left-shifted marrows, because immature cells are difficult or impossible to differentiate definitively into the appropriate cell lines. Consequently, and appropriately, M:E ratios may not be provided in histologic reports of left-shifted bone marrow samples.

(5) In one study, quantitative M:E ratios determined for histologic sections of marrow from healthy dogs were nearly twice those of cytologic preparations from the same dogs.[25] The cause of this discrepancy could relate to (i) inherent problems in identifying every cell in histologic sections, (ii) greater likelihood of sectioning through larger cells histologically (whereas each cell is either fully present or absent cytologically), or, possibly (iii) incomplete representation of the marrow in the nonparticulate thin regions evaluated in cytologic preparations.

(6) Increased M:E ratio: The M:E ratio (Figure 6.2B) may be increased because of myeloid hyperplasia, erythroid hypoplasia, or both. If marrow hematopoietic cellularity is increased, myeloid hyperplasia is contributing to the increased M:E ratio. If hematopoietic cellularity is decreased, erythroid hypoplasia is contributing to the increased M:E ratio. If the hematopoietic cellularity is not clearly increased or decreased, CBC results may help with interpretation. For example, concurrent neutrophilia would support granulocytic hyperplasia.

(7) Decreased M:E ratio: The M:E ratio (Figure 6.2A) may be decreased because of granulocytic (myeloid) hypoplasia, erythroid hyperplasia, or both. If the marrow hematopoietic cellularity is increased, erythroid hyperplasia is contributing to the decreased M:E ratio. If hematopoietic cellularity is decreased, granulocytic hypoplasia is contributing to the decreased M:E ratio. If the hematopoietic cellularity is not clearly increased or decreased, CBC results may help with interpretation. For example, concurrent regenerative anemia supports erythroid hyperplasia.

c. Maturation progression

(1) Because nearly all cells of the erythroid series can undergo mitosis (not metarubricytes), there should be many more of the late stages than the early stages (a so-called pyramidal distribution (Figures 3.1 and 6.4). There also should be synchronous nuclear and cytoplasmic maturation. As nuclei become smaller and chromatin denser, the cell size decreases, the cytoplasmic basophilia decreases, and the eosinophilic staining of hemoglobin increases.

(2) The granulocytic precursors are divided into two pools (Figures 2.2 and 6.4). The early pool (proliferation pool) contains cells capable of mitosis, with more myelocytes than myeloblasts or progranulocytes in health. Most granulocytes in the maturation pool are segmented neutrophils (forming the storage pool), with

band neutrophils being second or similar in abundance. There should be orderly and synchronous nuclear and cytoplasmic maturation throughout the lineage.

(3) The majority (> 80 %) of megakaryocytes are mature, with light blue and finely granulated cytoplasm similar to platelets; a minority are smaller with bluer cytoplasm and less nuclear lobulation. An increased number of immature forms usually indicates stimulated megakaryopoiesis and megakaryocytic hyperplasia. It may also occur with dysplastic diseases and megakaryocytic neoplasms.

d. Lymphoid cells (lymphocytes and plasma cells): Most of the lymphocytes in bone marrow of healthy animals are small, but few larger forms are present as well as low numbers of plasma cells. Some of the lymphoid cells reflect marrow lymphopoiesis and others reflect migration of lymphocytes to the marrow. Hematopoietic stem cells have the appearance of small lymphocytes but are expected to be present in very low (quantitatively insignificant) percentages.

3. Other cells: Macrophages are present in low percentages, usually even when increased, making quantitative assessment imprecise. Mast cells are rare. Stromal cells such as fibroblasts, osteoclasts, and osteoblasts are rare in cytologic preparations, but osteoblasts and osteoclasts may be more numerous when there is active bone growth or remodeling. The percentages and densities of nonhematopoietic cell types may be subjectively deemed increased, but decreases cannot be detected. Increased percentages can indicate or suggest certain pathologic processes.
4. Cell atypia or dysplasia: All cell types are evaluated for abnormal cytoplasmic and nuclear features. Abnormal features may reflect neoplastic or nonneoplastic alterations that are typical of certain pathologic processes or conditions.
5. Hemosiderin or stainable Fe
   a. Most of the Fe detected in bone marrow is in the form of hemosiderin within macrophages (or is from lysed macrophages in cytologic preparations). Unstained hemosiderin is golden brown. In routinely stained aspirate samples, hemosiderin takes up varying amounts of blue stain and appears yellow-green to brown or almost black (Figure 6.3B). A more complete assessment of Fe storage can be made if the samples are stained with an Fe stain such as Prussian blue. With such staining, additional forms of Fe also may be detected, e.g., Fe within sideroblasts (cytologically).
   b. The amount of Fe in the sample is estimated and reported by using a variety of terms (e.g., absent, small, moderate, or large amounts) or semiquantitative scoring.[28] Subjective amounts or scores may be interpreted as decreased, within limits of health, or increased. Decreased and increased amounts may suggest or support pathologic states. Although Fe evaluation is a routine part of bone marrow analysis, it is rarely the indication for it. Clinical information and blood findings (e.g., RBC appearance, reticulocyte hemoglobin content, and other RBC indices) are usually sufficient to identify (but not necessarily differentiate) clinical Fe deficiency and Fe restriction (see Chapter 3).
   c. Healthy cats lack detectable marrow Fe stores, but hemosiderin may be detected with hemolytic disorders, after a transfusion, and with myeloproliferative neoplasms.[29,30] Stores are not expected in healthy young animals (< ~1 yr of age) of any species.

C. Comparison of histologic and cytologic examinations
   1. Histologic examination of core samples (antemortem)
      a. Advantages, given good samples, good sample processing, and evaluation by an expert hematopathologist

(1) Better assessment of tissue architecture (how cells are arranged), necrosis, hemorrhage, infiltrative populations, focal neoplastic or inflammatory lesions, reactive lymphoid follicles, and myelofibrosis
(2) Better assessment of bone marrow hematopoietic cellularity
(3) Better assessment of mature megakaryocyte number and distribution; clustering occurs when megakaryopoiesis is increased or when the microenvironment is disrupted (e.g., with fibrosis)
(4) Assessment of the size and number of erythroid islands and therefore the erythroid "real estate," and thus improved recognition of erythroid hypoplasia and hyperplasia
(5) Direct assessment of the total myeloid or neutrophil "real estate" for evidence of myeloid hyperplasia or hypoplasia rather than combining estimates of hematopoietic cellularity and M:E ratio to reach such conclusions; however, the estimate is subjective and immature cell types may not be identifiable
(6) Detection of bone and vessel abnormalities, although these are typically of secondary interest compared to hematologic abnormalities; increased osteoclasts in an adult are a clue for hypercalcemic disorders
(7) Provides extra tissue for special stains and labeling

b. Disadvantages
(1) More expensive, requires anesthesia, and longer turnaround time
(2) Limited ability to differentiate cell types, especially immature cells
(3) Difficult to detect phagocytosis of cells
(4) Difficult or impossible to detect many dysplastic features
(5) Limited number of hematopathologists with strong training in the histologic evaluation of bone marrow from clinical patients

c. Potential problems with core samples include short samples of uncertain representation, samples consisting mostly of cortical and subcortical bone or blood clots, sample damage related to prior aspiration, crush artifact from traumatic collection (e.g., poor technique, dull needles), improper fixation, and thick sections or damage induced during sectioning.

2. Cytologic examination of aspirate samples
a. Advantages, given good samples, good sample processing, and evaluation by an expert hematopathologist
(1) Cytologic detail, allowing individual cells and cell populations to be differentiated and evaluated, enabling better assessment of the M:E ratio and maturation progression
(2) Better detection of phagocytosis (cells or organisms)
(3) Better detection of dysplastic or neoplastic cell features
(4) Little processing cost (most of the technique expense is in the collection procedure)
(5) Extra unfixed sample or smears for cytochemical staining and labeling, immunocytochemistry, flow cytometry (including use of antibodies that do not work on fixed tissues), or various PCR tests

b. Disadvantages
(1) Minimal ability to assess tissue architecture
(2) Difficult to detect necrosis and myelofibrosis
(3) Interpretation limited with collection of few or no marrow particles
(4) May be difficult to determine sample representation (i.e., to differentiate a poor sample from hypocellular bone marrow)

c. Potential problems with cytologic preparations include collection of extramedullary samples, "dry taps," excessive hemodilution, clotted samples, thick smears, lysed cells, and poor staining.

D. Postmortem bone marrow samples
1. Hematopoietic cells undergo autolysis rapidly because the insides of bones stay warm much longer than most tissues, and precursors of WBCs contain many proteolytic and lipolytic enzymes that enable the digestion of cells.
2. Samples should be collected as soon as possible after an animal's death so that they are adequate for evaluation (guideline: < 30 min for cytologic assessment). Histologic sections collected a day or more after death may have enough autolysis to prevent assessment of the neutrophil lineage; nuclei of later-stage cells may become round such that the cells resemble immature or less differentiated cells.
3. Samples should be collected from cancellous bone in the proximal humerus or femur, or from the axial skeleton (e.g., ilium, rib, or sternum). Although it is easier to collect samples from the diaphysis of long bones, these regions are not reliably representative of hematopoietic activity.
4. A paintbrush technique for collecting cells and brushing them onto slides is recommended for cytologic evaluation.[12] This technique does not provide particles for assessment, so paired histologic samples are critical to complete interpretation of the cytologic findings.

E. Flow cytometry and automated hematology analyzers (Sysmex XT-2000iV, ADVIA 2120) have been used to assess bone marrow aspirates and provide M:E ratios and partial differentials for clinical and toxicologic pathology samples, but they are not routinely used for clinical cases and cannot replace microscopic evaluation.[12,22,24,31–33]

BONE MARROW CLASSIFICATIONS

I. *Bone marrow hyperplasia* – from the Greek *hyper* (over) + *plasis* (formation) – is a term that indicates an increased number of nonneoplastic hematopoietic cells in bone marrow because of a stimulus that causes one or more cell lines to proliferate. The stimulus usually results from an increased need for cells but may occur secondary to aberrant production of stimulating cytokines (Table 6.3). A proliferation of neoplastic cells is neoplasia, not hyperplasia.

A. Erythroid hyperplasia
1. *Effective erythroid hyperplasia*: Epo stimulation of precursor cells leads to a proliferation of erythroid cells, erythroid hyperplasia, and increased release of reticulocytes, nRBCs, or RBCs into blood.
a. It is usually secondary to hemolytic (Table 3.12) or blood loss disorders (Figure 3.18). The mechanisms that lead to erythroid hyperplasia begin immediately after the onset of anemia, but the resultant erythroid hyperplasia may not be apparent until a few days later. The degree of erythroid hyperplasia should correspond to the duration and severity of the anemia.
b. *Secondary appropriate erythrocytotic disorders*: Persistent hypoxia or increased tissue demand for O_2 promotes Epo release, which causes erythroid hyperplasia and erythrocytosis (see Chapter 3 for disorders).
c. *Secondary inappropriate erythrocytotic disorders*: Inappropriate Epo production in the absence of hypoxia causes erythroid hyperplasia and erythrocytosis (see Chapter 3 for disorders).

Table 6.3. Disorders and conditions causing erythroid, myeloid, or megakaryocytic hyperplasia
Erythroid hyperplasia
Effective erythroid hyperplasia
Secondary to hemolytic or blood loss disorders*
Secondary appropriate erythrocytoses: right-to-left shunts (congenital or acquired), chronic pulmonary disease, hyperthyroidism
Secondary inappropriate erythrocytoses: renal neoplasms, cysts, or diseases; other neoplasms (hepatoma)
Ineffective erythroid hyperplasia
PIMA*
Nutritional deficiencies: Fe,* copper, folate, or cobalamin (vitamin B_{12})
Hemophagocytic syndrome
Congenital dyserythropoiesis of polled Herefords
Dyserythropoietic anemia and myopathy syndrome in English springer spaniels
Congenital dyserythropoietic anemia in Murray Grey calves
Myeloid (neutrophilic, granulocytic) hyperplasia
Effective myeloid hyperplasia
Inflammation,* infectious (bacterial, fungal, protozoal) or noninfectious (e.g, sterile foreign body, immune-mediated disease)
Other or unknown mechanisms
Paraneoplastic neutrophilia
Neutrophilia of leukocyte adhesion deficiency
G-CSF administration
Estrogen toxicosis (early)
Cyclic hematopoiesis in FeLV-infected cats
Ineffective neutrophilic hyperplasia
Immune-mediated neutropenia
Phenobarbital-induced neutropenia
Cobalamin deficiency, severe
Trapped-neutrophil syndrome (border collies)
Hemophagocytic syndrome
Megakaryocytic hyperplasia
Response to or recovery from thrombocytopenia:* withdrawal of myelosuppression or response to a disorder that causes decreased platelet survival (see Chapter 4)
Inflammation: infections, immune-mediated disorders, surgery, trauma

* A relatively common disorder, condition, or mechanism
Note: Information about eosinophilic, basophilic, mononuclear-phagocytic, lymphoid, mast cell, and generalized marrow hyperplasia is provided in the text. Lists of specific disorders or conditions are not complete but are provided to give examples.

d. *Primary erythrocytosis* of polycythemia vera is neoplasia, not hyperplasia. Inherited Epo receptor defects causing primary erythrocytosis and effective erythroid hyperplasia have not been reported in domestic mammals.

2. *Ineffective erythroid hyperplasia*: Erythroid hyperplasia occurs concurrently with a nonregenerative or poorly regenerative anemia.

a. Precursor-targeted immune-mediated anemia (PIMA) has been reported in dogs and cats, sometimes being lumped with PRCA.[34–37] It is also called nonregenerative immune-mediated anemia – a term that does not differentiate PIMA from preregenerative IMHA or PRCA – or nonregenerative IMHA, a term indicating hemolysis, which is often absent or minimal with PIMA. Like IMHA, canine PIMA is usually primary (not associated with underlying disease). Like primary IMHA in dogs, it is idiopathic.
   (1) PIMA is characterized by persistent nonregenerative anemia, marrow evidence of an erythroid response to anemia, and evidence of selective RBC precursor phagocytosis (*rubriphagocytosis*). Lack of detected rubriphagocytosis does not exclude PIMA; it may be undetected because it is absent, or it may be undetected when present because it is mild, the sample is not of good quality, or the examiner is not attuned to it. PIMA is considered immune mediated based largely on the apparent response to immunosuppressive therapy, selective rubriphagocytosis, and association with IMHA. When a dog has evidence of IMHA (e.g., spherocytosis, RBC agglutination, Coombs test positivity) but without reticulocytosis, it might have acute IMHA with a preregenerative anemia or a combination of IMHA and PIMA; either way, it has IMHA and appropriate treatment for IMHA can proceed without marrow evaluation. However, many dogs with PIMA are presented with a nonregenerative anemia and no clinical or laboratory evidence of hemolysis ("pure PIMA"); for these dogs, bone marrow evaluation is indicated.
   (2) Various bone marrow patterns may be present, apparently related to the stage of erythroid precursors that are targeted. There may be marked erythroid hyperplasia with a left shift or the appearance of maturation arrest with expansion of erythroid stages prior to the target-cell stage.[34,36] A left shift may not be apparent when late-stage precursors and/or reticulocytes are targeted. Myelofibrosis develops in many PIMA dogs and may become severe enough to limit hematopoiesis and mask typical PIMA findings. In most dogs with PIMA, even with myelofibrosis, rubriphagocytosis (rubriphagia) is evident and best seen in cytologic preparations (Figure 6.5),[37] though it may be seen in histologic sections as well. Myelofibrosis has also been reported in cats with PIMA.[35] Mild erythrophagocytosis in addition to rubriphagocytosis is common in the marrow of dogs with PIMA, even with early-stage PIMA and no laboratory evidence of hemolysis. It can be prominent in dogs with IMHA plus PIMA or after transfusions. Few rubriblasts contain single RBCs in rare dogs with PIMA (MAS observations).
   (3) When early erythroid cells are targeted (rubriblasts or prorubricytes), the erythroid lineage may be hypocellular and reported as erythroid hypoplasia, but expansion of early stages indicates an erythropoietic stimulus and hyperplastic response. If erythroid progenitor cells (prior to recognizable precursors) are the target of an immune-mediated attack, there may be PRCA (see Bone Marrow Classifications, Section II.B). Early-stage PIMA (with rubriphagocytosis and expanded early erythroid stages) and PRCA (with no or almost no erythropoiesis and no left shift or rubriphagocytosis) have been lumped together in many studies and references. It is unknown if there are significant clinical differences.
   (4) Unlike ineffective erythropoiesis caused by a nutritional deficiency, there is no definitive diagnostic finding for PIMA. Rather, PIMA is supported by an appropriate constellation of findings including, retrospectively, response to

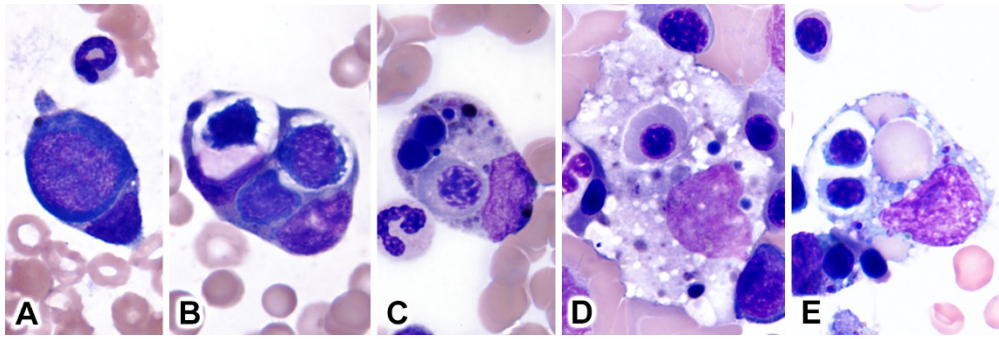


Figure 6.5. Bone marrow from dogs with PIMA showing rubriphagocytosis of a rubriblast (A), prorubricytes to early rubricytes (B), a later rubricyte (C), and a metarubricyte (D) by normal-appearing macrophages (each macrophage nucleus is lower-right in the cell). Blue degraded cellular material is also apparent in B–D. Rarely, as shown here (E), polychromatophils are a major target of destruction along with late-stage erythroid precursors (but not mature RBCs).

therapy. MDS may be a differential in some cases, but findings and clinical outcomes in dogs with suspected PIMA rarely support MDS in the author's (MAS) experience. In contrast, MDS with erythroid predominance is more common in cats and a major differential for cats with erythroid hypercellularity and persistent nonregenerative anemia, especially where FeLV infections are more prevalent.

b. Nutritional deficiencies
   (1) Fe-deficiency anemia: In the Fe-deficient state, anemia develops despite erythroid hyperplasia. Erythropoiesis is defective, late-stage erythroid precursors do not mature properly, and RBCs are not released adequately into the blood. Reticulocytosis may be present, but the release of reticulocytes is not enough to meet the need. As Fe deficiency progresses, rubricytes may appear smaller with less cytoplasm and hemoglobin.
   (2) Cobalamin deficiency (rare):[38,39] Cobalamin deficiency secondary to inherited abnormalities of intestinal cobalamin receptors has been reported in young giant schnauzers, beagles, border collies, Australian shepherds, and komondors. Dysplastic changes may be present in erythroid and myeloid lineages. Hypocobalaminemia was the suspected cause of pancytopenia in an adult cat with presumed gastrointestinal disease, but the marrow had generalized hypoplasia rather than a pattern of ineffective hematopoiesis.[40] Neither metronidazole-induced myelosuppression (with delayed recovery after metronidazole withdrawal) nor another myelosuppressive insult were excluded.
   (3) Copper or folate deficiency in domestic mammals might cause or contribute to ineffective hematopoiesis, but naturally occurring cases are not well documented.

c. Hemophagocytic syndrome, which is characterized by nonselective phagocytosis, may cause or contribute to anemia secondary to a wide variety of disorders (see Bone Marrow Classifications, Section I.B.2.e, regarding neutropenia). Nonselective hemophagocytosis appears to be more common in cats than in dogs.

d. Congenital dyserythropoiesis of polled Herefords (see Chapter 3)[41]
e. Dyserythropoietic anemia and myopathy syndrome of English springer spaniels[42,43]
f. Congenital dyserythropoietic anemia of Murray Grey calves (see Chapter 3)[44]

B. *Myeloid hyperplasia*: Unless stated otherwise, myeloid hyperplasia is characterized by an increased number of neutrophil precursors and therefore includes *granulocytic hyperplasia* and more specifically *neutrophilic hyperplasia*. Any of these terms may be used. Other forms of granulocytic hyperplasia are eosinophilic hyperplasia and basophilic hyperplasia (rare). Monocytic hyperplasia may also be recognized, often concurrently with neutrophilic hyperplasia, but early stages of neutrophil and monocyte precursors are not easily differentiated, so monocytic hyperplasia may or may not be recognized when present with neutrophilic hyperplasia.
1. *Effective myeloid hyperplasia*: Continual stimulation of neutrophil precursors by G-CSF, GM-CSF, or certain interleukins leads to granulocytic (and neutrophilic) hyperplasia, increased release of neutrophils to blood, and typically neutrophilia with or without a left shift (see Chapter 2). Other cytokines may promote effective eosinophilic or basophilic hyperplasia, but the latter would typically have minimal impact on the M:E ratio.
   a. Inflammation: Neutrophilic hyperplasia is typically caused by an inflammatory process that results from any of a wide variety of infectious and noninfectious disorders (see Chapter 2).
   b. Other or unknown mechanisms of neutrophilic hyperplasia are listed in Table 6.3 or discussed in Chapter 2. Note: Myeloid hyperplasia reported in neutropenic FeLV-infected cats with cyclic hematopoiesis led to resolution of neutropenia, supporting that it was effective rather than ineffective, despite occurring with neutropenia.
2. *Ineffective neutrophilic hyperplasia*, *ineffective granulocytic hyperplasia*, *ineffective myeloid hyperplasia* (Figure 2.9E): This form of hyperplasia is recognized by neutropenia with concurrent neutrophilic hyperplasia. There may be a myeloid left shift or apparent maturation arrest. Similar patterns may be seen with certain neoplasms (MDS, AML), but that is neoplasia, not hyperplasia.
   a. Immune-mediated neutropenia including marrow-targeted immune-mediated neutropenia, seen mostly in dogs and rarely suspected in cats:[45–47]
      (1) Neutrophilic hyperplasia results from persistent proliferation of neutrophil precursors in response to cytokines, but neutropenia occurs from destruction of neutrophil precursors or neutrophils before or soon after release from bone marrow. There may or may not be a marrow left shift or appearance of a maturation arrest, depending on the targeted neutrophil stage. When mid- to early-stage neutrophil precursors are targeted, the myeloid lineage may be normocellular to hypocellular and suggestive of and reported as hypoplasia, but expansion of early stages indicates a hematopoietic stimulus and hyperplastic response. Expansion of early myeloid stages has been great enough to be misinterpreted as AML. Phagocytosis of neutrophils may be detected in bone marrow, liver, or spleen. Neutrophil production might be effective, at least in some cases, but with rapid peripheral neutrophil destruction causing the appearance of ineffective production.
      (2) Anti-neutrophil antibodies targeting precursors or mature neutrophils are thought to mediate cell destruction directly (IgG) or via complement opsonization. Although assays for antibodies on neutrophils or their precursors have provided evidence of this, analytical and diagnostic validation studies are very limited in veterinary medicine.[48]

(3) Cell-mediated mechanisms may be involved in some cases, such as neutropenia associated with marrow lymphocytosis of neoplastic or nonneoplastic granular lymphocytes in dogs.[49–51] Small granular lymphocytes with scant cytoplasm are mildly to markedly increased in a subset of dogs with immune-mediated neutropenia and ineffective myeloid hyperplasia. In some cases, PARR testing has shown clonality and therefore support for a neoplasm of granular lymphocytes expressing CD3 and CD8.[51] The median time to resolution of neutropenia after chemotherapy in dogs with these granular lymphocyte neoplasms was 15 wk. In addition to such cases, the author (MAS) has seen several cases of ineffective myeloid hyperplasia with marrow small granular lymphocytosis that responded quickly to immunosuppressive glucocorticoid therapy and appeared to resolve, although neither PARR nor long-term follow-up was done to assess for an underlying indolent neoplasm.

(4) Immune-mediated neutropenia has been suspected but is not well documented in cats.[52] For example, a young cat with a progressive FeLV infection had marked neutropenia that progressed to persistent pancytopenia which appeared rapidly responsive (within 3 d) to prednisolone, initially and again after relapse. A bone marrow aspirate revealed myeloid hyperplasia and phagocytosis of neutrophils and RBCs. Although the cytopenias were steroid responsive, suggesting immune-mediated disease, their pathogenesis may have involved FeLV, hemophagocytic syndrome, or specific immune-mediated cytophagia.

b. Phenobarbital-induced neutropenia: Isolated neutropenia or, more commonly, multiple cytopenias (often pancytopenia) occur in some dogs given phenobarbital for seizure disorders, typically after a few months of therapy.[53–55] Neutropenia is associated with myeloid (neutrophilic) hyperplasia, often with a left shift and increased marrow phagocytosis of myeloid cells that mimics (and may reflect) immune-mediated neutropenia. Neutropenia most commonly resolves within 2–3 wk of discontinuing phenobarbital and switching to a different anticonvulsant (without instituting immunosuppressive therapy). Similar changes of ineffective hematopoiesis may be seen in the erythroid lineage, and dysplastic cells may be present. When thrombocytopenia is present, megakaryocytes are not reduced and may be hyperplastic. Immune-mediated cell destruction may contribute to the cytopenias, but the cause is not known. Primidone may have similar effects.[54]

c. Severe cobalamin deficiency (rare): Ineffective neutropoiesis may occur in dogs with cobalamin malabsorption because of mutations in genes for proteins forming the intestinal cobalamin receptor; cobalamin is needed for hematopoiesis.[39,56,57] Dysplastic features may be present in multiple lineages. Such mutations have been documented in giant schnauzers, Australian shepherds, border collies, beagles, and komondors. The dogs respond quickly to parenteral cyanocobalamin therapy.

d. Trapped-neutrophil syndrome: Border collies with this inherited autosomal recessive condition have facial and limb developmental abnormalities and secondary infections associated with neutropenia or low-normal neutrophil concentrations despite concurrent neutrophilic hyperplasia (see Chapter 2 for more information).[58] Despite the name of the disorder, neutrophils can reach other tissues and cause suppurative inflammation.

e. Hemophagocytic syndrome (suspected):[59–63] Neutropenia may be one of multiple cytopenias (anemia, bicytopenias, pancytopenia) associated with *hemophagocytic syndrome*, the nonselective phagocytosis of mature or developing hemic cells by

nonneoplastic macrophages stimulated by cytokines in response to a variety of infectious or noninfectious inflammatory or neoplastic diseases. Inflammatory disorders include immune-mediated disease, so nonselective phagocytosis could occur with selective phagocytosis, thus complicating a diagnosis. Myeloid (or erythroid) hyperplasia with or without a left shift may be present along with phagocytosis of various hemic cells by normal-appearing macrophages.[64] In some suspected cases, hemophagocytosis has been more apparent in the spleen than in the bone marrow.[64]

(1) Hemophagocytic syndromes are not well described in veterinary medicine and proposed diagnostic criteria have not been rigorously evaluated.[62,65,66] There is no definitive diagnostic test for hemophagocytic syndrome, so other causes of cytopenias must be excluded. Consideration must be given to erythrophagocytosis associated with hemorrhage and phagocytosis of neutrophils at sites of neutrophilic inflammation.

(2) Nonselective hemophagocytosis in various organs seems to be more common in cats than dogs, with or without accompanying cytopenias (author's observation, MAS). When hemophagia is present, it supports that it may be a contributing cause of cytopenias, but other mechanisms may be as important.[65]

(3) Human primary and secondary hemophagocytic syndromes include life-threatening hemophagocytic lymphohistiocytosis and macrophage activation syndrome.[67]

(4) Hemophagocytic syndrome is distinct from hemophagocytic histiocytic sarcoma. Neutropenia is not expected with canine hemophagocytic histiocytic sarcoma.

f. Feline immunodeficiency virus (FIV) or FeLV infections may cause ineffective myelopoiesis in cats, including MDS.

3. *Eosinophilic myeloid hyperplasia* is characterized by increased numbers of eosinophils and eosinophil precursors in bone marrow.
   a. Specific mediators, including interleukin 5 (eosinophil differentiation factor) and GM–CSF from mast cells, macrophages, and lymphocytes, stimulate eosinopoiesis.
   b. Eosinophilic hyperplasia may or may not be recognized in animals with eosinophilia (Table 2.13), and blood eosinophilia may not be present when mild eosinophilic hyperplasia is present; the rate of eosinophil migration from blood to other tissues may be greater than the rate of egress from bone marrow to blood.
   c. Cats with MDS may have marrow eosinophilic hypercellularity with or without blood eosinophilia;[68,69] however, these eosinophils and those accompanying other clonal stem cell neoplasms may be neoplastic rather than hyperplastic increases.
4. *Basophilic myeloid hyperplasia* is characterized by increased numbers of basophils and basophil precursors in bone marrow. This type of hyperplasia is rarely recognized but might be found in animals with basophilias (Table 2.15).
5. *Monocytic hyperplasia* is a hyperplastic state that is present when monocytic precursors are increased in the bone marrow. It usually is not seen as a primary bone marrow finding; more often, it is found concurrently with granulocytic hyperplasia, particularly when there is a macrophagic or granulomatous component to the inflammatory process.

C. *Megakaryocytic hyperplasia*, a nonneoplastic increase in megakaryocytes, occurs when megakaryopoiesis (and thrombopoiesis) are persistently stimulated. Platelet concentrations vary from decreased to increased. Megakaryocytes may have increased pleomorphism and dysplastic features.
   1. With thrombocytopenia: In response to thrombocytopenia, stimuli (stem cell factor, thrombopoietin, interleukin 3, and GM–CSF) promote proliferation of megakaryocytes

to replace the missing platelet mass. The thrombocytopenia frequently is caused by disorders that decrease platelet survival. When thrombocytopenia is caused by a myelosuppressive agent or process, there may be rebound megakaryocytic hyperplasia after removal of the suppression.

2. With thrombocytosis: Mild megakaryocytic hyperplasia may be found concurrently with one of the several causes of reactive thrombocytosis, including inflammation and neoplasms.
3. Adequate or increased megakaryopoiesis does not necessarily indicate there is adequate thrombopoiesis. For example, in addition to mediating platelet destruction, many antibodies from human patients with immune-mediated thrombocytopenia bind to megakaryocytes and impair proplatelet formation and thrombopoiesis without impairing megakaryopoiesis.[70–72]

D. *Generalized bone marrow hyperplasia* is present when there is concurrent granulocytic, erythroid, and megakaryocytic hyperplasia. It can be seen whenever there are concurrent stimuli for proliferation of neutrophils, RBCs, and platelets, such as in some cases of IMHA in which destructive or consumptive thrombocytopenia and inflammatory neutrophilia are present. When present, causes for hyperplasia of each lineage should be considered separately, as the main stimuli for the different lineages are mainly distinct from each other. Generalized hyperplasia should not be confused with the hypercellularity of polycythemia vera, a neoplastic (not hyperplastic) state that may cause concurrent erythrocytosis, leukocytosis, and thrombocytosis.

E. Some pathologists use the term *relative hyperplasia* when one or more lineages appear increased compared to others, but there is either hyperplasia or there is not. A lineage may be increased relative to another but be hypoplastic if the marrow cellularity is reduced. Hematopoietic cellularity must be known to conclude there is hyperplasia; if it is not known, no such conclusion can be made, and an increased M:E ratio could reflect myeloid hyperplasia, erythroid hypoplasia, both, or myeloid and erythroid hyperplasia with more myeloid hyperplasia than erythroid hyperplasia.

II. *Bone marrow hypoplasia* – from the Greek *hypo* (under) + *plasis* (formation) – is a nonspecific term that is commonly used to indicate a decreased age-adjusted number of one or more hematopoietic cell lines in the bone marrow. However, only some of these decreases are truly hypoplasia and caused by (i) decreased cell production from the absence of stimulating agents, (ii) the presence of inhibitors, or (iii) direct damage to progenitor cells. Other decreases in a cell lineage may be caused by precursor cell destruction concurrent with increased but ultimately ineffective hematopoietic activity, thus yielding hypocellular findings that differ from hypoplasia because of a left shift or maturation arrest. As is true of hyperplasia, hypoplasia may be generalized or selective (Table 6.4).

A. *Generalized marrow hypoplasia* (panhypoplasia), a concurrent decrease in granulocyte, erythroid, and megakaryocyte precursors, is typically recognized when cytopenias develop. When severe, with little or no evidence of hematopoiesis, the terms *aplastic anemia* or *aplastic pancytopenia* may be applied, the latter being more descriptive of the usual state (pancytopenia), and the former reflecting reliance on the hematocrit to recognize decreased hematopoiesis when aplastic anemia was first described in people (aplastic anemia is generalized hypoplasia or aplasia, not PRCA).

1. Generalized marrow hypoplasia or aplasia is most reliably identified with a good core bone marrow biopsy sample. In established aplastic anemia, medullary spaces contain mostly univacuolated adipocytes, while in acute myelosuppression from infectious or

Table 6.4. Disorders and conditions that cause hypoplastic bone marrow states
Generalized marrow hypoplasia
Immune-mediated myelosuppression, autoimmune or alloimmune (bovine neonatal pancytopenia)
Infections:* chronic *Ehrlichia canis* infection, parvoviral infection, or associated with fungal (e.g., histoplasmosis) or protozoal (e.g., leishmaniasis, cytauxzoonosis) myelitis
Toxicants/drugs: albendazole, azathioprine, bracken fern, cephalosporins, many chemotherapeutic agents, colchicine, endogenous or exogenous estrogen (dogs), fenbendazole, griseofulvin, trichothecene mycotoxins, phenylbutazone, trichloroethylene-extracted soybean meal (historical), trimethoprim-sulfadiazine
Irradiation: whole body, environmental; focal irradiation may cause focal hypoplasia
Gelatinous transformation of bone marrow: severe malnutrition
Myelophthisis: neoplasms,* collagen myelofibrosis, osteopetrosis, osteosclerosis
Marrow necrosis: ischemia, infections, acute irradiation
Selective erythroid hypoplasia or aplasia
Pure red cell aplasia:* primary immune-mediated, secondary to recombinant human Epo treatment, FeLV subgroup C infection, possibly canine parvovirus FeLV infections
Anemia of inflammatory disease* (mild hypoplasia)
Renal disease (chronic)*
Endocrine disorders: hypothyroidism, hypoadrenocorticism, hypoandrogenism, hypopituitarism (rare) (mild hypoplasia except for hypopituitarism)
Drug induced: chloramphenicol (suprapharmacologic doses)
Cyclic hematopoiesis (grey collies, grey-collie crosses, other breeds)
Selective granulocytic or neutrophilic hypoplasia or aplasia (rare)
Infections (may cause generalized hypoplasia but affect neutropoiesis the most)
Toxicants/drugs: azathioprine (cats), bracken fern (ptaquiloside, early stage), chloramphenicol (cats), griseofulvin (cats), phenytoin (diphenylhydantoin); may appear to be present with some causes of generalized hypoplasia
Immune-mediated: pure white cell aplasia
Other: cyclic hematopoiesis (grey collies, other breeds), possibly G-CSF deficiency
Selective megakaryocytic hypoplasia or aplasia
Idiopathic amegakaryocytic thrombocytopenia (may be immune mediated)
Toxicants/drugs: possibly dapsone, ribavirin; may cause generalized hypoplasia but affect neutropoesis the most, e.g., early estrogen toxicity

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

toxic injury, matrix may predominate until recovery or replacement by first multivacuolated and later univacuolated adipocytes. In some conditions, hypoplasia or aplasia may be associated with necrosis, gelatinous transformation of bone marrow, or myelofibrosis. A false impression of marrow aplasia or hypoplasia can be obtained when marrow is sampled from inactive sites (e.g., not proximal enough in the humerus or femur, particularly in older animals) or when cores are tangential and yield mostly hypocellular (fatty) subcortical marrow that may not be representative of hematopoiesis.

2. Many disorders or conditions have been associated with generalized bone marrow hypoplasia or aplasia,[73,74] but identifying and proving the cause of the disorder is frequently difficult or impossible. An insult to the bone marrow may have occurred weeks before clinical illness.
   a. Fat-filled aplastic or severely hypoplastic bone marrow (aplastic anemia): Major causes to consider are immune-mediated myelosuppression (well characterized in human but not veterinary medicine), chronic *Ehrlichia canis* infection (dogs), and chemical/drug toxicosis that occurred at least several days earlier such that there has been time for adipocytes to replace the hematopoietic tissue. However, many cases are idiopathic, i.e., no cause is determined. In such cases, immunosuppressive therapy ± bone marrow stimulants (e.g., the thrombopoietin receptor agonist eltrombopag) may be administered;[75] subsequent clinical recovery may be coincidental or due to the therapy.
   b. Severe bone marrow hypoplasia associated with increased matrix or plasma (acute myelosuppression): Major causes to consider are acute hematopoietic cell depletion by a viral infection (parvovirus infection in dogs or cats), chemical/drug toxicosis, or immune-mediated destruction (bovine neonatal pancytopenia caused by colostral alloantibodies induced by vaccination or possibly other antigen sources).[76–78] Irradiation is an uncommon cause. With time, marrow may recover (rebound hematopoiesis) or be replaced by adipocytes.
   c. Hypoplasia may also occur secondary to myelitis caused by fungal or protozoal infections, in which case marrow findings are characterized primarily by inflammatory infiltrates, not increased fat or matrix.
   d. Myelosuppression from drugs may be predictable (e.g., chemotherapeutic agents that target dividing cells) or idiosyncratic, the latter occurring unpredictably (at least with available information) in a low percentage of patients receiving a drug at therapeutic dosages.
      (1) With enough exposure, predictable myelosuppression occurs with bracken fern (*Pteridium* spp.) ingestion in cattle, trichothecene mycotoxins in feeds,[79] many chemotherapeutic agents, azathioprine, and chronic exogenous or endogenous hyperestrogenism in dogs, the latter occurring with certain ovarian or testicular neoplasms.[80,81]
      (2) Idiosyncratic myelosuppression has been suspected with several drugs, but proof of causation is difficult. Incriminated drugs include albendazole, phenylbutazone, trimethoprim-sulfadiazine, griseofulvin, fenbendazole, and methimazole.[82–86]
      (3) Dogs and cats with aberrant genes for P-glycoprotein (multidrug resistance protein 1) are susceptible to myelosuppression induced by drugs that are normally excreted by this transport protein.[87] These drugs include vincristine, vinblastine, and doxorubicin. The defect can occur in any breed, but it is most common in herding and some sighthound breeds; it is reportedly present in > 50 % of collies, Australian shepherds, and long-haired whippets. Border collies are similarly sensitive but for a different unknown reason.[88]
   e. Whole-body radiation exposure (therapeutic or environmental exposure to X-rays, gamma irradiation, or beta irradiation) can cause systemic marrow hypoplasia, and focal marrow hypoplasia can occur at focal sites of irradiation. However, little or no myelosuppression was apparent in CBCs of dogs with various cancers of the head/neck, pelvis/perineum, trunk, or extremities treated with 48–62 Gray of definitive

fractionated external beam megavoltage ionizing radiotherapy daily or on alternate days over several weeks.[89]

f. Other causes of hypoplasia are typically recognized by accompanying findings.
   (1) Gelatinous transformation of bone marrow: generalized hypoplasia with decreased adipocytes and increased eosinophilic or pale basophilic matrix (acid mucopolysaccharides) that stains blue with Alcian blue, pH 2.5; this occurs with severe malnutrition, e.g., starvation or chronic disease
   (2) Myelophthisis (marrow replacement): hemic neoplasms, myelofibrosis, or increased bone (osteosclerosis or osteopetrosis); myelofibrosis and osteosclerosis are particularly associated with PIMA in dogs, and may occur with certain other chronic erythropoietic stimuli (e.g., in pyruvate kinase deficiency), hemic neoplasms, or after inflammation and/or necrosis
   (3) Necrosis: areas of coagulative or liquefactive necrosis, with or without associated inflammation, from ischemia, infections, or acute irradiation; antemortem collection artifact may be confused with necrosis but lacks evidence of cell death

g. Inherited causes of marrow aplasia/hypoplasia should be considered in neonates but are rarely recognized in domestic mammals.[90]

B. *Selective erythroid hypoplasia or aplasia* is a pathologic state in which the mass of erythroid precursors is decreased but the granulocytic and megakaryocytic cell lines are not. All erythroid stages are decreased proportionately when fewer progenitor cells enter into recognizable erythropoiesis. In contrast, although the term hypoplasia may be applied to the erythroid hypocellularity seen in some dogs with early-stage PIMA, expansion of early erythroid precursors (rubriblasts) in such cases indicates an erythropoietic stimulus and hyperplastic response despite subsequent precursor destruction.[36]

1. *PRCA* is a pathologic state in which there is selective erythroid aplasia or severe hypoplasia. It leads to normocytic, normochromic, nonregenerative anemia.
   a. *Primary PRCA* is considered an immune-mediated disorder; it may be seen concurrently with immune-mediated spherocytic hemolytic anemia. It is unclear if some published studies of canine PRCA included dogs with early-stage PIMA; PIMA and PRCA are often lumped together. Sera of some dogs reported to have PRCA had a demonstrable serum substance (probably antibody) that inhibits erythropoiesis in vitro.[91] An immune-mediated mechanism in many FeLV-negative affected cats is supported by response to immunosuppressive therapy. Affected dogs and cats are often young, and marrow lymphocytosis may be prominent,[29,34,92] which is also true for dogs with early-stage PIMA.[36] Increased eosinophil precursors in the marrow is not uncommon. Concurrent megakaryocytic aplasia has been reported in a young cat.[93]
   b. *Secondary PRCA* has an associated cause.
      (1) It has occurred secondary to treatment with recombinant human Epo in dogs,[94–96] horses,[97,98] and cats; antibodies generated against human Epo apparently cross-react with endogenous Epo and impair erythropoiesis.
      (2) Although chronic *Ehrlichia canis* infections are associated with generalized bone marrow hypoplasia, PRCA was reported in one infected dog.[99]
      (3) Selective erythroid hypoplasia or aplasia can occur in cats infected with FeLV subgroup C, which arises in some cats infected with FeLV-A. FeLV-C is not horizontally transmissible like FeLV-A. FeLV-C enters target cells via heme transporters, which are present in erythroid progenitors and precursors (and other cell types). Its presence can impair erythroid development from

progenitors such that PRCA and nonregenerative anemia occur several weeks after infection.[100–105] Regressive or latent FeLV infections also might be responsible for PRCA or erythroid hypoplasia in some cats.[106] In such cats, proviral DNA is incorporated within genomes of hemic or nonhemic marrow cells where it may alter cell functions, but virus is not actively produced so immunoassays for FeLV antigens (p27) are negative. The viral DNA can be detected by PCR analysis, sometimes in bone marrow when blood PCR testing is negative. Additionally, viral reactivation and viremia may occur in immunocompromised cats. Positive results of any FeLV test support but do not prove that FeLV caused erythroid hypoplasia in a particular cat.

c. Although AML and MDS may give the appearance of selective erythroid hypoplasia relative to myeloid hypercellularity, the neoplastic proliferation of myeloid cells causes decreased production of functional nonneoplastic myeloid cells in addition to erythroid cells, and therefore causes a more-generalized hypoplasia.

2. Anemia of inflammatory disease (see Chapter 3): Decreased erythropoiesis is part of the pathogenesis of this nonregenerative anemia. Contributors are thought to include cytokine-mediated inhibition of erythropoiesis and decreased Fe availability. Erythroid hypoplasia may not be recognized because it is mild or because concurrent granulocytic hyperplasia makes it difficult to determine whether there is an absolute decrease in erythroid precursors. However, the presence of nonregenerative anemia in the absence of erythroid hyperplasia is evidence of suppressed (not appropriately expanded) erythropoiesis even when erythroid hypoplasia is not clearly present.
3. Chronic renal disease with decreased renal functional mass results in insufficient Epo production and thus mild to at least moderate erythroid hypoplasia. Impaired marrow response to Epo and effects of decreased renal excretion of hepcidin may also contribute to the erythroid hypoplasia (see Chapter 3).
4. Selective erythroid hypoplasia caused by endocrine disorders: The nonregenerative anemias of hypothyroidism, hypoadrenocorticism, and hypoandrogenism are caused by erythroid hypoplasia, but the degree of hypoplasia in bone marrow samples can be mild and not recognized in routine bone marrow examinations. The presence of nonregenerative anemia in the absence of erythroid hyperplasia is evidence of suppressed (not appropriately expanded) erythropoiesis. In contrast, erythroid hypoplasia was severe in a 2-wk-old puppy with a rare condition of congenital, adenohypophyseal pars distalis developmental arrest and findings consistent with central hypothyroidism.[107]
5. Drugs: Suprapharmacologic doses of chloramphenicol can induce transient erythroid hypoplasia in dogs[108] and cats.[109] There may be concurrent myeloid and megakaryocytic hypoplasia in cats.
6. Cyclic hematopoiesis: Grey collies, grey collie crosses, and rare other dogs with this autosomal recessive disorder have cyclic erythroid hypoplasia but only mild anemia because periods of hypoplasia are much shorter than the RBC lifespan.[110,111]

C. *Selective granulocytic or neutrophilic hypoplasia* (Table 6.4) is a pathologic state in which the number of granulocyte or specifically neutrophil precursors is decreased without generalized marrow hypoplasia. There are not enough eosinophil and basophil precursors in health for a decrease in these lines to cause overall granulocytic hypoplasia or even to be recognized as hypoplastic lineages. Although the term hypoplasia may be applied to the myeloid hypocellularity seen with immune-mediated or drug-induced neutropenia associated with apparent myeloid maturation arrest, expansion of early myeloid stages in such conditions indicates a cytokine stimulus and a hyperplastic response.

1. Several infections and drugs have been reported to cause selective granulocytic hypoplasia, but many of them have been associated with hypoplasia of other cell lines and are not actually selective. There may be the appearance of selective granulocytic hypoplasia when there is undetected damage to other cell lines or if neutropenia appears first because of neutrophil kinetics (short life span). This may happen in parvoviral infections, where neutropenia may be caused by a combination of damage to cells with mitotic potential, depletion of neutrophil pools because of excessive tissue demand, endotoxin-induced damage to marrow cells, increased margination, and rapid emigration from blood to inflamed tissue. It may also occur with drugs or toxicants such as azathioprine.[112]
2. Animals with precursor-targeted immune-mediated neutropenia can have myeloid hypocellularity accompanied by a myeloid left shift (early-stage to mid-stage precursor destruction) that may be interpreted as selective neutrophilic hypoplasia, but expansion of the early precursors reflects a hyperplastic response to cytokine stimulation. The early myeloid expansion is sometimes great enough to mimic AML.
3. *Pure white cell aplasia (PWCA)*, a very rare condition recognized by severe unexplained neutropenia with an absence or near absence of granulocyte precursors, was diagnosed in a dog with neutropenia and very few granulocyte or monocyte precursors in the bone marrow.[46] Antibody-mediated cell destruction was suspected. Similar findings could occur with antibody- or cell-mediated inhibition of either progenitor cells or granulocyte-monocyte colony-stimulating factor (GM-CSF).[113]
4. Cyclic hematopoiesis (see Chapter 2): Grey collies, grey collie crosses, and other dogs with this autosomal recessive disorder caused by mutations in *AP3B1*, the gene for adaptor-related protein complex 3 subunit beta 1, have cyclic myeloid hypoplasia and neutropenia that is out of synchrony with the hematopoietic cycles of other lineages.[110,111]
5. Chronic idiopathic neutropenia caused by G-CSF deficiency: Similar to precursor-targeted immune-mediated neutropenia, the bone marrow in the Rottweiler with this disorder had a decreased M:E ratio with many myeloblasts and progranulocytes, but myelocytes and later neutrophil stages were rare. Using a bioassay, patient G-CSF was undetectable when the dog was neutropenic and maximally stimulated to produce G-CSF, and evidence of serum inhibitors (e.g., antibodies) of bone marrow culture assays was lacking. Immunosuppressive therapy was provided for only 4 d and no G-CSF trial was done. The disorder was attributed to G-CSF deficiency.[114]
6. Release of segmented neutrophils from the neutrophil storage pool and band neutrophils from the maturation pool in response to acute, severe inflammatory stimuli may decrease the M:E ratio and give the appearance of neutrophilic hypoplasia when in fact neutropoiesis is being stimulated and neutrophilic hyperplasia may develop.

D. *Selective megakaryocytic hypoplasia or aplasia* (Table 6.4) is a rare pathologic state in which the number of megakaryocytes is decreased, but the erythroid and granulocytic cell lines are not. Congenital causes recognized in people have not been reported in domestic mammals.
   1. Rare cases of idiopathic amegakaryocytic thrombocytopenia have been reported in dogs and are often presumed to be immune-mediated.[115]
   2. Megakaryocytic hypoplasia may be disproportionately prominent in some cases of generalized hypoplasia caused by toxicants, but truly selective hypoplasia is rarely reported. It has been reported with ribavirin administration to cats, accompanied by megakaryocytic dysplasia and erythroid hypoplasia at the highest dose.[116] Amegakaryocytic thrombocytopenia was attributed to dapsone therapy in a dog.[117] Megakaryocytic hypoplasia occurs with reduced erythropoiesis early in canine estrogen toxicity, prior to myeloid hypoplasia.[118]

E. Some use the term *relative hypoplasia* when one or more lineages appear decreased compared to others, but there is either hypoplasia or there is not. A lineage may be decreased relative to another, but still be hyperplastic if the marrow cellularity is increased. Hematopoietic cellularity must be known to conclude there is hypoplasia; if it is not known, no such conclusion can be made.

III. *Bone marrow lymphocytosis* is a neoplastic (see Bone Marrow Classifications, Section IX.C) or nonneoplastic accumulation of lymphocytes in the bone marrow. Nonneoplastic lymphocytosis may occur from inflammatory infiltration or local lymphoid hyperplasia. In tissue sections of bone marrow, the lymphoid cells may occur in unorganized aggregates or nodules, in germinal centers, or as a diffuse population.

A. Because bone marrow is a primary and secondary lymphoid tissue, inflammatory disease may cause lymphoid hyperplasia in bone marrow as well as in lymph nodes and spleen. The hyperplastic lymphoid cells are typically small lymphocytes, but larger forms and plasma cells may be prominent.

B. The reported percentage of marrow nucleated cells that are lymphocytes in healthy domestic mammals is quite variable, but it is usually < 10 %, extending up to about 20 % in some feline and canine studies.[1,2,6,13,24,119]

C. A variety of disorders that involve stimulation of the immune system may cause bone marrow lymphocytosis. Examples include canine ehrlichiosis[5] and systemic lupus erythematosus.[120] When bone marrow lymphocytosis in cats was defined as > 16 % lymphocytes with > 50 % hematopoietic cellularity, it was associated most commonly with immune-mediated anemia and PRCA.[119] Lymphocytes were mostly B lymphocytes and were often present in aggregates, but they may be distributed diffusely as well. Marrow lymphocytosis is also seen in dogs with PRCA and early-stage PIMA, and in a subset of dogs with immune-mediated neutropenia, in which case granular lymphocytes may be prominent.

D. Neoplastic lymphocytosis of the bone marrow can occur with lymphocytic leukemia, lymphoblastic leukemia, or with lymphoma. Lymphoma typically arises in other tissues (primary bone marrow lymphoma is rare). Cell numbers and marrow distributions vary from patchy to diffuse. Immunophenotyping or PARR testing may be indicated.

E. When there is granulocytic and erythroid hypoplasia, the lymphocyte population may appear more prominent without being increased (only relatively increased), particularly with aspirate samples for which the density of lymphocytes per unit area of bone marrow cannot be assessed as it can in histologic sections.

IV. *Bone marrow mastocytosis* is a neoplastic (see Bone Marrow Classifications, Section IX.B.5) or nonneoplastic accumulation of mast cells in the bone marrow. Nonneoplastic accumulations may be called mast cell hyperplasia; the mast cells likely accumulate by locally increased mast cell production secondary to homing of mast cell progenitor cells, not by increased production of mast cells through mast cell divisions. Bone marrow mastocytosis has been reported in dogs with aplastic anemia,[121] regenerative anemia, Fe-deficiency anemia, hypoplasia secondary to a Sertoli cell tumor, and lymphoma.[122] It occurs in few dogs with PIMA (authors' observations). It probably represents a reactive state associated with inflammation.

V. Myelofibrosis

A. *Myelofibrosis* is a pathologic state of increased fibrous connective tissue in the bone marrow (Figure 6.6).

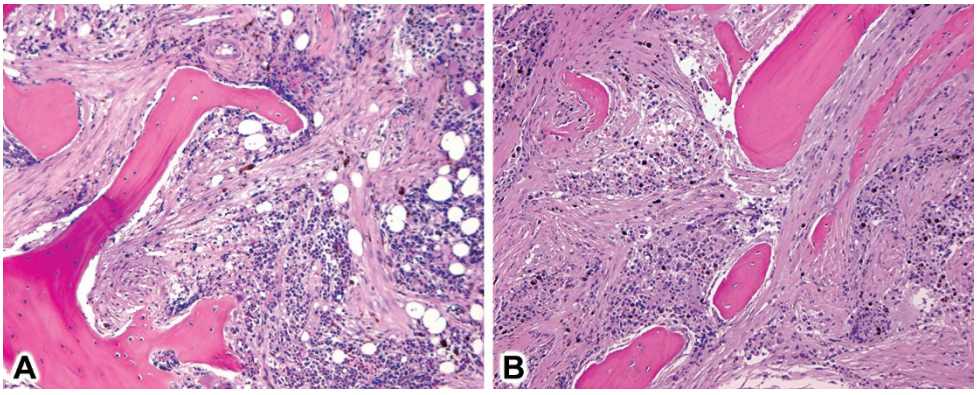


Figure 6.6. Histologic sections of bone marrow from two dogs with PIMA and associated collagen myelofibrosis. Paratrabecular streams of pale pink collagen and fibroblasts extend into medullary spaces and replace hematopoietic tissue. Fibrosis is more severe in B where abnormal small islands or thin fingers of new bone have formed in addition to the expected larger trabeculae; this osteosclerosis is common when collagen myelofibrosis occurs. Hematoxylin and eosin stain.

1. *Reticulin* myelofibrosis is the deposition of type 3 collagen and requires special stains or immunolabeling for detection. Even though reticulin is a type of collagen, reticulin fibrosis is not referred to as collagen myelofibrosis.
2. *Collagen* myelofibrosis is the deposition of type 1 collagen, and it can be seen with hematoxylin and eosin staining of tissue sections unless it is mild; detection and confirmation can be done with special stains (Masson's trichrome). It is often associated with new bone formation and increased trabeculae.
3. Most publications about myelofibrosis in people are in regard to reticulin myelofibrosis. It is typically graded for severity in areas of active hematopoiesis.[21] Once collagen bundles are present in human myelofibrosis, myelofibrosis has reached the highest grade, but collagen myelofibrosis may be graded from mild to marked in veterinary medicine.

B. Myelofibrosis can be primary (see Myeloproliferative neoplasms) or secondary, and it is idiopathic when the cause is unknown. In the author's experience (MAS), collagen myelofibrosis appears to be secondary in the vast majority of affected dogs, most often to PIMA.[36] The mutations driving human primary myelofibrosis were not found in a study of dogs with collagen myelofibrosis.[123]

C. Reticulin and collagen myelofibrosis commonly occur in dogs with PIMA, varying from reticulin myelofibrosis to mild, moderate, and severe, effacing collagen myelofibrosis.[34,36] Many reports of so-called idiopathic myelofibrosis may have been secondary to PIMA. In the author's experience (MAS), the diagnosis of PIMA in many dogs with severe collagen myelofibrosis is supported by persistent nonregenerative anemia, evidence of ineffective erythropoiesis with rubriphagocytosis, and, in some, response to immunosuppressive therapy. However, when collagen myelofibrosis is severe and effacing, evidence of PIMA or another cause may be lacking, in which case the myelofibrosis must be labeled idiopathic. With extensive myelofibrosis, a deficiency in all hematopoietic lineages may develop and cause aplastic anemia (pancytopenia).

D. Collagen myelofibrosis and osteosclerosis develop in dogs with regenerative anemias from inherited pyruvate kinase deficiency.[124,125] Experimentally, dogs given high doses of recombinant human erythropoietin also developed collagen myelofibrosis.[126]

E. Other causes of collagen myelofibrosis include focal or widespread marrow damage and bone marrow neoplasms that cause production of mediators of fibroplasia. Such neoplasms include MDS, which is the diagnosis offered by some pathologists for cases others diagnose as PIMA based on selective rubriphagocytosis and, retrospectively, response to immunosuppressive therapy. Moderate or severe collagen myelofibrosis was also reported in three anemic, epileptic dogs treated with phenobarbital, and it was accompanied by either coagulative necrosis or individual cell death of an unreported degree.[127]

F. In people, myelofibrosis (reticulin or collagen types) can be caused by autoimmune disorders, certain chemical toxicants (benzene), irradiation, various osteopathies (hypovitaminosis D, renal osteodystrophy), infections (tuberculosis), and various primary and metastatic bone marrow neoplasms.[21]

VI. Myelitis

A. *Myelitis*, in the context of hemic disorders, is bone marrow inflammation. Some people use the word *osteomyelitis* to differentiate bone marrow myelitis from spinal cord myelitis, whereas others use the word to indicate concurrent bone inflammation and bone marrow inflammation.

B. There are several agents that can cause myelitis; the inflammatory populations vary with the agent.[128]

1. Fungal myelitis (e.g., histoplasmosis, blastomycosis, aspergillosis)
2. Protozoal myelitis (e.g, leishmaniasis, cytauxzoonosis) (Figure 6.7A)
3. Bacterial myelitis, including mycobacteriosis

C. Inflammation may also be present in the bone marrow secondary to necrosis from any cause, and macrophages may be increased secondary to immune-mediated disorders or marrow neoplasms.

VII. Bone marrow necrosis

A. Criteria for establishing the presence of bone marrow necrosis vary, as do the extent and relative significance of necrosis when it is reported. The term *bone marrow necrosis* may be restricted to bone marrow samples in which necrosis is the primary and predominant abnormality, or it may be applied when necrosis is not the major pathologic process.

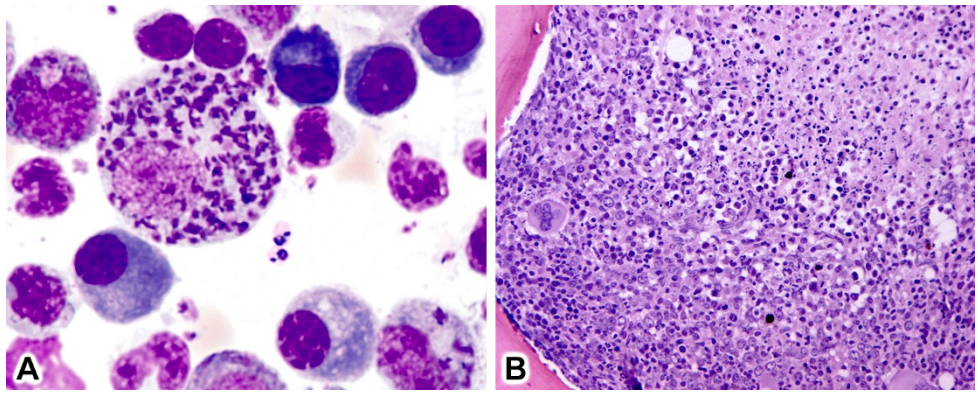


Figure 6.7. A. Amastigotes of *Leishmania*, extracellular and within bone marrow macrophages; rod-shaped kinetoplasts are most apparent in the two nearly central extracellular amastigotes. There is also lymphoplasmacytic inflammation. B. Histologic section of canine bone marrow with necrosis in the upper right quadrant; note karyorrhectic cells, hematoxylin and eosin stain.

Necrosis is distinct from apoptotic cell death, though some authors have referred to apoptosis as necrosis.

B. Bone marrow necrosis (Figure 6.7B) must be differentiated from collection and processing artifacts, both cytologically and histologically.
   1. Cytologically, staining and preservation artifacts can mimic cell death, as can cell trauma and lysis during smearing.
   2. Crush artifact, plasma pools, and edema may appear similar to necrosis in histologic sections of bone marrow, but pyknotic cells and reactive inflammation that occur with necrosis are absent. Fibrin may be present in a core because of clotting secondary to a prior aspirate in the same region.

C. Bone marrow necrosis has been reported in association with many disorders, including toxicants, bacterial sepsis, *Ehrlichia canis* infections, canine parvovirus infection, feline panleukopenia, feline infectious peritonitis, bovine virus diarrhea in calves, systemic lupus erythematosus, lymphoma, disseminated intravascular coagulation, and hypoxia.[1,127] However, the extent of the necrosis within a sample has not always been reported, the extent in the animal is unknown, and the clinical significance of the finding is generally unknown. In one retrospective canine study, coagulative necrosis or individual cell death were seen in a variety of conditions (e.g., sepsis, epilepsy, systemic lupus erythematosus) and in association with several drugs (e.g., chemotherapeutic agents, phenobarbital, carprofen, and metronidazole).[127]

D. Bone marrow necrosis has been considered idiopathic when unassociated with an underlying disease or drug exposure.[127] In one study, eight of nine dogs classified as having idiopathic bone marrow necrosis had only individual cell death rather than coagulative necrosis, and the extent of cell death was not reported.[127] These dogs also had marked anemia (sometimes with neutropenia or thrombocytopenia), mild to marked myelofibrosis, and sometimes a prominent macrophage population. Neither the disorder nor the significance of cell death in these cases is clear.

VIII. Myelophthisis

A. Strictly speaking, *myelophthisis* means "marrow wasting" (*myelo-* plus *-phthisis*) or wasting of the spinal cord.

B. The term is commonly used to indicate failure of the bone marrow to produce cells because of replacement or displacement of bone marrow by abnormal tissue. Causes of myelophthisis include neoplastic cell proliferation, myelofibrosis, osteosclerosis, and osteopetrosis. If the disease process affects most bone marrow sites, animals develop anemia, neutropenia, and thrombocytopenia.

IX. Bone marrow hemic cell neoplasms

A. General concepts and terms
   1. Neoplasms of bone marrow hemic cells are not as common as many other neoplasms in domestic mammals. The general diagnosis of hemic cell neoplasms is not difficult when there are numerous poorly differentiated cells in a blood or bone marrow sample but establishing the cell of origin can be difficult. It also can be difficult to differentiate neoplasms of well-differentiated hemic cells (e.g., chronic leukemias) from inflammatory conditions. Specific diagnoses may be reached by a combination of exclusionary tests, immunophenotyping, cytochemical reactions, bone marrow examination, and molecular genetic testing (Figure 6.8). However, diagnostic conclusions are limited by the great diversity of hemic neoplasms combined with their rarity and limited validated testing across domestic mammalian species.

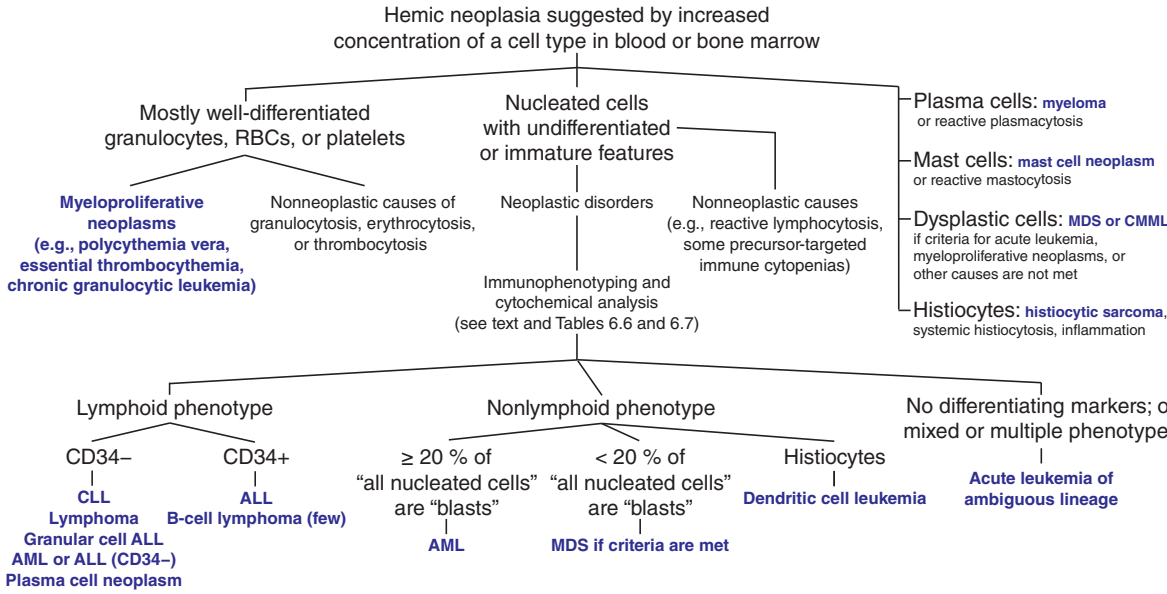


Figure 6.8. General algorithm for hemic neoplasms. Such neoplasms may be suspected with mild to marked increases in well-differentiated or atypical cells in blood or bone marrow.

Top left: An increased concentration of well-differentiated RBCs, platelets, lymphocytes, monocytes, neutrophils, eosinophils, or basophils could be neoplastic or nonneoplastic.

- Nonneoplastic disorders or conditions include appropriate or secondary inappropriate erythrocytosis, reactive thrombocytosis, or various types of inflammation (see Chapters 2–4).
- Exclusion of nonneoplastic disorders incriminates a MPN such as chronic myeloid leukemia or polycythemia vera (Table 6.5), but confirmatory cytogenetic tests may be unavailable.

Figure 6.8. *continued*

Top right: Plasma, mast, histiocytic, or dysplastic cells may be neoplastic or nonneoplastic.

- If the cells are clearly plasma cells, reactive plasmacytosis (inflammation) must be differentiated from a plasma cell myeloma or plasma cell leukemia (neoplastic).
- If the cells are clearly mast cells, reactive mastocytosis (inflammation) must be differentiated from a metastatic mast cell neoplasm or mast cell leukemia.
- If dysplasia is prominent and the cells do not fulfill criteria for AML, then MDS or MDS/MPN should be considered along with nonneoplastic causes of dysplasia.
- Bone marrow histiocytes may be inflammatory or neoplastic and can be further characterized as dendritic cells or macrophages.

Middle and bottom: An increased population of nucleated cells with undifferentiated and immature features may be neoplastic or nonneoplastic. Clonality testing may be useful when a lymphoid neoplasm is suspected but unclear; a positive result supports a neoplasm.

- Nonneoplastic immature populations may increase mildly in blood or at least moderately in bone marrow with reactive lymphocytosis, a severe inflammatory reaction, reactive megakaryocytic hyperplasia, or markedly stimulated erythropoiesis or granulopoiesis associated with acute anemia, early-stage precursor-targeted immune-mediated cytopenias, or early rebound hematopoiesis after myelosuppressive injury.
- Neoplastic populations in blood or bone marrow may be identifiable by a combination of microscopy and panels used for immunophenotyping and cytochemical phenotyping.
  - Left: A lymphoid phenotype and CD34 positivity supports ALL (few B-cell lymphomas also express CD34), whereas a CD34-negative population of lymphocytes may occur with CLL (maturity may not be clear microscopically), lymphoma, ALL of granular lymphocytes, in cases of ALL or AML when CD34 is not expressed, or with neoplasms of plasma cells that were phenotyped because they lacked clear features of plasma cells.
  - Center: If cells have a nonlymphoid phenotype, they may be identifiable as megakaryoblasts, rubriblasts, monoblasts, myeloblasts, or myelomonoblasts. Once nonneoplastic conditions have been excluded as the cause of an increased population of undifferentiated, immature, or dysplastic myeloid cells, WHO criteria for myeloid neoplasms may be used to determine the type of neoplasm.
    - If a differential cell count of a bone marrow aspirate indicates that at least 20 % of ANC are blasts (see definitions below), that supports AML; the cell type indicates the specific type of AML. Other criteria must be met for specific subtypes, such as ≥ 50 % of the nucleated cells being megakaryocytic for acute megakaryoblastic leukemia. A diagnosis of pure erythroid leukemia requires ≥ 30 % rubriblasts and ≥ 80 % erythroid cells (i.e., M:E ≤ 1:4).
    - If blasts account for < 20 % of ANC, MDS may be present. Prominent dysplasia and ineffective hematopoiesis with cytopenias supports MDS, but specific criteria must be met and nonneoplastic causes of these abnormalities must be excluded.
    - Rarely, cells have a dendritic phenotype indicative of a dendritic cell leukemia.
  - Right: If atypical cells do not express specific identifying markers, it is evidence for an acute undifferentiated leukemia or other rare acute leukemia of ambiguous origin including those consisting of more than one distinct atypical population (e.g., lymphoid blasts and myeloid blasts) or of one population of atypical cells expressing multiple phenotypes (e.g., lymphoid and myeloid markers or mixed B and T lymphoid markers).

ANC, all nucleated cells, includes granulocytic, monocytic, erythroid, and megakaryocytic cells, and excludes lymphocytes, plasma cells, macrophages, and mast cells.
Blasts refers to myeloblasts, megakaryoblasts, and monoblasts ± promonocytes (if there is monocytoid differentiation), but not rubriblasts or lymphoblasts.
ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CMML, chronic myelomonocytic leukemia; and MPN, myeloproliferative neoplasm

2. Characterization and classification of hemic neoplasms in domestic mammals[68,129–134] (Table 6.5) has generally followed developments in understanding and classification of similar human neoplasms, many of which are now defined by specific genetic mutations.[135–137] The human model has provided useful insights into the types of hemic neoplasms that may occur in domestic animals, but substantial investigation is needed in veterinary medicine to reach similar degrees of characterization.
3. Bone marrow hemic cell neoplasms may arise in the bone marrow (e.g., acute myeloid leukemias) or spread to the bone marrow (e.g., lymphoma).
4. The term *leukemia* is from the Greek *leukos* ("white") + *haima* ("blood") = "white blood" and refers to an increased buffy coat layer. However, neoplastic hemic cells do not have to be in the blood for leukemia to be present; the proliferation may be exclusively in the bone marrow. It may also start in the spleen, rather than the marrow, and spread to the blood. And it is important to recognize that not all hemic neoplasms originating in the bone marrow are classified as leukemia. For example, MDS, plasma cell myeloma, and some myeloproliferative neoplasms such as essential thrombocythemia are not leukemias.
5. *Acute leukemia* is a proliferation of relatively less differentiated hemic cells with an acute (brief) clinical course, often even when treated with current protocols. Many or all of the neoplastic cells are blasts with dispersed chromatin and often nucleoli and cytoplasmic basophilia. Cell immaturity often makes it impossible to determine lineage microscopically, and it can be difficult or impossible to determine lineage (even lymphoid from myeloid) with all available tools. CD34 expression supports classification as an acute leukemia, either lymphoid or myeloid, but acute leukemias may be CD34 negative, and other cells can express CD34. For example, a minority of B-lymphocyte lymphomas have a recognizable expression pattern that includes few to many CD34-positive cells.[138] Because of extensive proliferation of acute leukemia cells in bone marrow, multiple cytopenias of nonneoplastic cell lines are common.[139,140]
6. *Chronic leukemia* is a slower proliferation or accumulation (decreased apoptosis) of relatively well-differentiated hemic cells that are similar in appearance to mature cells seen in health (e.g., neutrophils or lymphocytes). An animal may live for many months after diagnosis, even without treatment.
7. The term *myeloproliferative disease* has been used generally to refer to any proliferative neoplastic disorder of nonlymphoid hemic cells, including neutrophils, eosinophils, basophils, monocytes, RBCs, megakaryocytes, and perhaps mast cells. However, it was also used more specifically in a restricted context referring to those clonal conditions previously classified as *chronic myeloproliferative diseases* and now classified as *myeloproliferative neoplasms* (see Bone marrow hemic cell neoplasms in the following section B.4).
8. *Lymphoproliferative disease* refers to a proliferation of lymphocytes, usually implying a neoplastic proliferation. *Lymphoproliferative neoplasia* (lymphoid neoplasia) clarifies that the proliferation is not reactive inflammation and encompasses any neoplastic proliferation of any type of lymphoid cell, including plasma cells. Note: In some conditions, the major reason for increased lymphocyte numbers may actually be decreased cell death rather than increased cell proliferation.
9. Poorly differentiated myeloid cells (blasts) can appear lymphoid (lymphocyte-like), and lymphocytes can appear myelomonocytoid, so while microscopy often provides critical clues about cell types, other tests may be needed to determine lineage. Even then, the lineage of neoplastic cells may be unclear.

Table 6.5. Major classifications of veterinary hemic cell neoplasms involving blood or bone marrow[a]

Classification	Proliferating cell lineage
Lymphoid neoplasms[b]	Lymphocyte
Acute lymphoblastic leukemia/lymphoma	Precursor B or T lymphocyte
Chronic lymphocytic leukemia	Mature B, T, or NK lymphocyte
Lymphoma*	B, T, or NK lymphocyte
Plasma cell myeloma	Plasma cell (from B lymphocyte)
Myeloid neoplasms	Nonlymphoid hemic cell
Acute myeloid leukemia (AML), not otherwise specified[c]	Nonlymphoid hemic cell
AML with minimal differentiation (M0)	Granulocyte
AML without maturation (M1)	Granulocyte
AML with maturation (M2)	Granulocyte
Acute myelomonocytic leukemia (M4)	Granulocyte and monocyte
Acute monoblastic/monocytic leukemia (M5)	Monocyte
Pure erythroid leukemia	RBC
Acute megakaryoblastic leukemia (M7)	Megakaryocyte
Acute basophilic leukemia	Basophil
Myeloproliferative neoplasms[c]	Nonlymphoid hemic cell
Chronic myeloid leukemia (BCR-ABL translocation)	Granulocyte
Chronic neutrophilic leukemia	Neutrophil
Chronic eosinophilic leukemia	Eosinophil
Polycythemia vera	RBC ± WBC and megakaryocyte
Essential thrombocythemia	Megakaryocyte
Unclassifiable (includes chronic basophilic leukemia)	Nonlymphoid hemic cell
Myelodysplastic/myeloproliferative neoplasms[d]	Nonlymphoid hemic cell
Chronic myelomonocytic leukemia	Neutrophil/monocyte
Atypical chronic myeloid leukemia	Granulocyte
Myelodysplastic syndromes[d]	WBC, RBC, or megakaryocyte
Mastocytosis	Mast cell
Metastatic mast cell neoplasms (cutaneous or visceral)	Mast cell
Mast cell leukemia	Mast cell
Histiocytic neoplasms	
Histiocytic sarcoma	Dendritic cell or macrophage
Dendritic cell leukemia	Dendritic cell
Acute leukemia of ambiguous lineage[d]	Unknown, mixed, or multiple

* A relatively common disorder or condition

[a] Guided by the WHO and International Consensus Classification of human hematologic malignancies with omission of the many types that are rare and/or have not been documented in domestic mammals (e.g., those classified by specific genetic abnormalities)[135–136]

[b] Subtypes of lymphoid neoplasms have been omitted.

[c] The "M" abbreviation system is not part of the current WHO nomenclature for AML. Human AMLs historically classified as acute promyelocytic leukemia (M3) are now categorized as *AML with recurrent genetic abnormalities*, and those historically classified as erythroleukemia (M6) are classified as (i) pure erythroid leukemia, (ii) a different AML, or (iii) MDS.

[d] See the text for subtype information omitted from this table.

10. *Blast cells* are small to large cells with immature nuclear features and replicative potential. They typically have smooth or finely stippled chromatin and lack features of mature or differentiated cells.
    a. Myeloblasts, monoblasts, rubriblasts, and megakaryoblasts are relatively large precursor cells with basophilic cytoplasm and visible nucleoli.
    b. The term *lymphoblast* has been defined and used in different ways.
        (1) It may refer to a relatively small precursor cell that will become a mature lymphocyte; such cells are of small to intermediate size and lack increased cytoplasmic basophilia and prominent nucleoli.
        (2) It has also been used to refer to relatively large lymphocytes that have transformed from mature lymphocytes through antigenic stimulation; such "blast-transformed" cells grew larger, became bluer from increased cytoplasmic mRNA, and developed immature nuclear features including prominent nucleoli.
            (a) Although developmentally not lymphoid precursor cells, these cells will divide to form new lymphocytes.
            (b) These cells appear similar to myeloblasts, monoblasts, rubriblasts, and megakaryoblasts. When such cells are seen in blood smears, they may be lymphoid or myeloid and are commonly classified as blasts, even though they do not resemble precursor lymphoblasts.
    c. CD34 expression supports the blastic precursor nature of hemic cells, and therefore classification of a leukemia as acute (see Acute leukemia above in Section IX.A.5).
    d. Note that the term *erythroblast* refers to any nRBC in the erythroblast system of nomenclature (Table 6.2), including metarubricytes, which are precursors but lack mitotic potential (a typical blast trait).

B. Myeloid neoplasms: Classification of myeloid neoplasms in dogs and cats was described in 1991 by the ALSG, a multi-institutional group of veterinary clinical pathologists using human classification systems that have since been modified.[68,135,141,142] It is also addressed in a publication of the Armed Forces Institute of Pathology (AFIP) and the WHO (AFIP/WHO classification) as part of a series on histologic classification of tumors of domestic animals.[130] However, further investigations are needed to define appropriate classifications and diagnostic criteria for myeloid neoplasms in domestic animals.[129] Until that occurs, the ALSG, AFIP/WHO, and human classifications can be used as guidelines to understand the types of myeloid neoplasms that may affect veterinary patients.[135–137]
    1. *AMLs* are rare, rapidly progressive acute leukemias involving nonlymphoid WBC, erythroid, megakaryocytic, or undifferentiated precursors, with *differentiation* referring to commitment to a particular lineage. Clinical signs are generally nonspecific, but enlarged lymph nodes are common and can be mistakenly attributed to lymphoma, even after cytologic or histologic evaluation. Circulating blasts are reported in the vast majority of cases, and concurrent cytopenias are common.[133] Diagnosis requires microscopic evaluation of blood and bone marrow aspirates (Figure 6.9) by a veterinary hematology expert, and often immunophenotyping and cytochemical testing. Cytogenetic testing may become important in veterinary medicine, as it is in human medicine.
        a. AML has been reported in dogs,[133,140,143,144] cats,[143,145,146] and few horses,[147,148] as well as rarely in cattle[149] and alpacas.[150] Advances in understanding and species-specific diagnostic tools, best developed for dogs, are improving recognition of these disorders, but many obstacles exist.

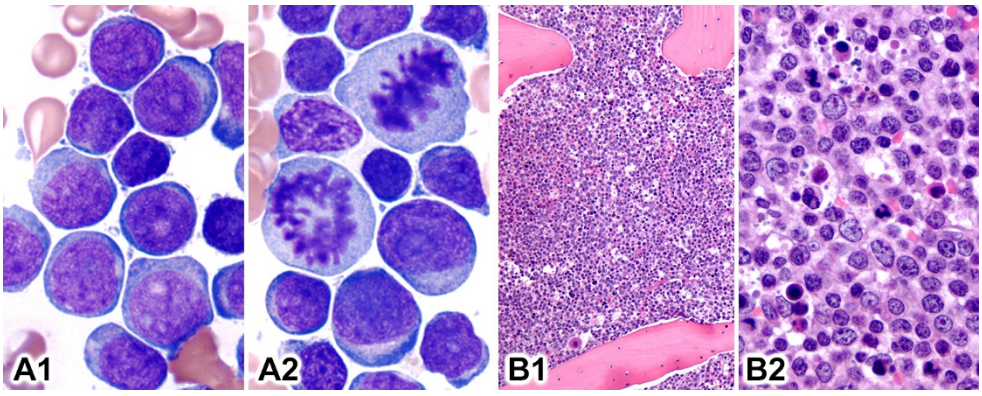


Figure 6.9. A. Undifferentiated blasts including mitotic cells (A2) in the bone marrow aspirate of a dog with AML. B. Histologic sections of marrow from a different dog with AML showing typical hypercellularity and replacement (myelophthisis) by a diffuse proliferation of neoplastic cells accompanied by macrophages containing degraded cells (B2), hematoxylin and eosin stain.

b. In the human classification scheme that veterinary medicine generally uses as a guide, the diagnosis of AML requires that blasts account for > 20 % of "all nucleated cells" in the bone marrow or blood, except when a specific genetic mutation is identified that defines a leukemia as AML. Blasts are defined as myeloblasts, megakaryoblasts, monoblasts (plus promonocytes when monocyte differentiation is evident); "all nucleated cells" does not include plasma cells, lymphocytes, macrophages, or mast cells. The blast percentage decision threshold was previously 30 %, which is what the ALSG adopted for domestic mammals. However, human conditions with 20–29 % blasts (previously MDS rather than AML) behave clinically like those with 30 % or more blasts (AML) (Figure 6.8), so the threshold was changed to 20 %; that has been largely adopted in veterinary medicine. Veterinary-specific studies relating blast percentages to outcome are lacking.[151] Note: Blast percentages may be inaccurate or imprecise for many reasons, so values near the decision limit should not be interpreted strictly.
c. Most human AML categories currently do not directly apply to veterinary medicine because they are based on specific clonal genetic abnormalities (translocations or inversions) that define the neoplasm but that have not been identified as recurrent genetic abnormalities in veterinary patients. This includes many cases of AML that arise in people with current or previous MDS-related cytogenetic abnormalities. Similarly, therapy-related AMLs arising months to years after use of such alkylating agents, radiation, and topoisomerase inhibitors have not been recognized in veterinary species.
d. Instead, most veterinary AMLs fall into the category of *AML, not otherwise specified*, which is used for AMLs that do not fit into any of the other categories, including when genetic information is lacking.[135]
   (1) Classification is based mostly on lineage(s) and the degree of maturation, where *maturation* refers to microscopic structural changes associated with development of cell functions (Table 6.5). Immunophenotyping and cytochemical reactions are important tools to determine lineage.

(2) The 2016 WHO guidelines brought a major change in approach to classifying those myeloid neoplasms with > 50 % of the marrow nucleated cells being erythroid precursors. This pattern is particularly relevant to cats. Instead of the previous determination based on blasts (myeloblasts, monoblasts, megakaryoblasts) as a percentage of all nonerythroid cells, the percentage of blasts remains as for all other AMLs and is based on "all nucleated cells." Consequently, the blast percentage would now be lower, and many cases previously classified as erythroleukemia are classified as MDS (< 20 % blasts) with the updated criteria. When > 80 % of the cells are erythroid precursors (an arbitrary cutoff), ≥ 30 % are rubriblasts (also an arbitrary cutoff), and < 20 % are myeloblasts, criteria are met for a diagnosis of pure erythroid leukemia (previously called DiGuglielmo disease, acute erythremic myelosis, true erythroleukemia, M6-Er, or M6B).[152]

e. Rarely, an AML presents as a *myeloid sarcoma* (chloroma), a discrete mass of any AML subtype that occurs with or without blood and bone marrow involvement.

2. Myeloid neoplasms include *acute leukemia of ambiguous lineage*, for which blast cells lack identifying characteristics and can appear lymphoid. This category includes (i) bilineal leukemias consisting of two distinct blast populations, (ii) combined myeloid and lymphoid leukemias, (iii) undifferentiated acute leukemias lacking lymphoid or myeloid markers, and (iv) biphenotypic acute leukemias with lymphoid and myeloid markers.

3. *Myelodysplastic syndromes* (MDSs), now also called *myelodysplastic neoplasms* by the WHO, are a group of hemic clonal neoplasms characterized by ineffective hematopoiesis, usually one or more persistent (≥ 4 mo) cytopenias, ≥ 10 % dysplastic cells in one or more lineages, and/or an increase in blasts to a degree less than that seen in AML.[135–137,151] The cells have impaired maturation and survival. Involvement of multiple cell lines is caused by neoplastic transformation of multipotential hematopoietic stem cells. Leukemia develops in a substantial percentage of domestic mammals and human patients with MDS, so MDS is considered a preleukemic condition. FeLV can induce MDS (and AML) in cats.[153]

a. Since a basic veterinary MDS classification system was proposed by the ALSG in 1991,[68] there have been several proposed modifications and categories,[129,130,151,154–156] and the guiding human MDS classification systems have varied and evolved.[136,137] Diagnostic criteria for human subtypes involve genetic abnormalities, number of cytopenias, number of dysplastic cell lines, percentage of blasts, or the percentage of ring sideroblasts (nRBCs with a ring of stainable Fe around the nucleus). Appropriate classifications and criteria for MDS in domestic species are not clear, but otherwise unexplained dysplasia or increased blasts form the basis for the diagnosis. Infectious, toxic, nutritional, immune-mediated, hereditary, and neoplastic conditions other than MDS must be excluded. There is no definitive test.

b. To be classified as a MDS, blasts (defined as myeloblasts, megakaryoblasts, monoblasts ± promonocytes) can account for up to only 20 % (30 % according to ALSG guideline) of all marrow nucleated cells (excluding plasma cells, lymphocytes, macrophages, and mast cells); with ≥ 20 % blasts (previously ≥ 30 % blasts), the diagnosis would be AML (Figure 6.8). With some human classification systems, an MDS/AML category has been created for cases with 10–19 % blasts in the marrow, emphasizing that MDS and AML are on a continuum of clinical behavior.[137]

(1) It should be noted that the percentage of blasts in rare cases of precursor-targeted immune-mediated anemia or neutropenia can be as high as those in MDS and AML, so the entire constellation of laboratory and clinical findings must be considered to reach an accurate diagnosis.
(2) In addition, blast percentages may be inaccurate because a sample is not representative, too few cells are differentiated to characterize a representative sample, or many cells are disproportionately lysed or unevenly distributed in areas that can be assessed.
(3) Even if the results are accurate, it is likely that clinical behavior is not consistently different between a myeloid neoplasm with, for example, 18 % blasts and one with 22 % blasts. Aggressive clinical behavior may indicate that a neoplasm is an AML rather than a MDS despite a reported blast percentage < 20 %.

c. *Dysplasia* is microscopic atypia reflecting abnormal cell maturation. Human WHO diagnostic criteria for MDS, except for MDS with excess blasts, require at least 10 % of the cells in any lineage to have dysplastic features. Dysplastic changes that may be observed in MDS include the following:[10,68,154]
(1) *Dysmyelopoiesis* (Figure 6.10A) includes giant cell size, abundance of azurophilic granules, hypogranular cytoplasm, polyploid or hypersegmented neutrophil nuclei, Pelger–Huët cell features (hyposegmentation), and binucleation.
(2) *Dyserythropoiesis* (Figure 6.10B) includes multiple nuclei, nuclear fragmentation, nuclear budding, nuclear lobulation, megaloblastic appearance, excessive cytoplasm relative to the nucleus, abnormal distribution of siderosomes, macrocytosis, unequal nuclear division, and atypical Howell–Jolly bodies.
(3) *Dysmegakaryopoiesis* (Figure 6.10C) and *dysthrombopoiesis* include nonlobed large nucleus, multiple small nuclei, dwarf megakaryocytes (micromegakaryocytes), megaplatelets, and abnormal cytoplasmic granularity or vacuolation of platelets.

d. The diagnosis of MDS is complicated by substantial interindividual variation in identification of dysplasia, and by the occurrence of similar amounts of dysplasia in

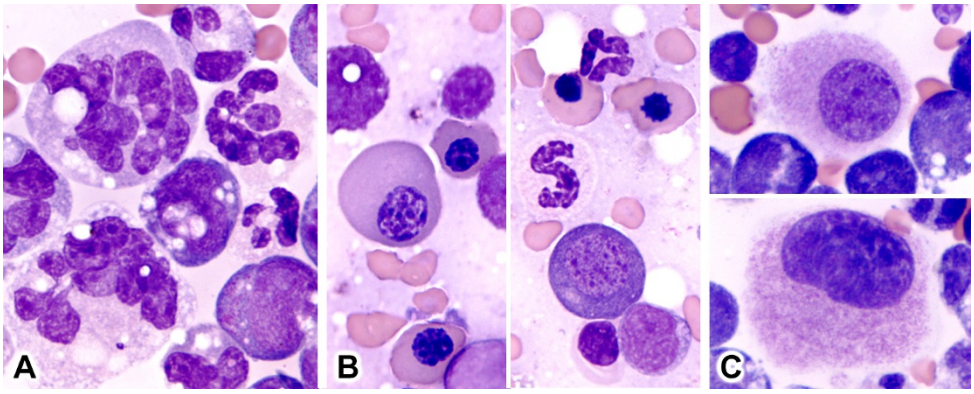


Figure 6.10. Dysplastic large hypersegmented myelomonocytic cells (A), erythroid precursors with asynchronous maturation and megaloblastic forms (B), and micromegakaryocytes with hyposegmented nuclei and mature cytoplasm (C), feline bone marrow aspirates.

nonneoplastic cytopenic conditions. When significant dysplasia is present, MDS must be differentiated from nonneoplastic congenital dyshematopoiesis (dysmyelopoiesis) disorders:[155]

(1) Hereditary poodle marrow dyscrasia (miniature and toy poodle macrocytosis) (see Chapter 2)
(2) Malabsorption of cobalamin (vitamin B_{12}) in giant schnauzers, border collies, Australian shepherd dogs, beagles, and komondors (see Chapter 3)
(3) Dyserythropoietic anemia and myopathy syndrome in English springer spaniels (see Chapter 3)
(4) Congenital dyserythropoiesis and progressive alopecia of polled Hereford calves (see Chapter 3)
(5) Congenital dyserythropoietic anemia in Murray Grey calves (see Chapter 3)
(6) Dysthrombopoiesis associated with macrothrombocytopenia in congenital platelet disorders including tubulin defects of Cavalier King Charles spaniels and some terrier breeds (see Chapter 4)
(7) Pelger–Huët and related anomalies; megakaryocyte hypolobulation has been described in dogs and cats, in addition to granulocyte and monocyte hypolobulation (see Chapter 2)

e. MDS must also be differentiated from *acquired secondary dyshematopoiesis* disorders in which dysplastic changes are neither congenital nor clonal.[157] Dysplastic features should resolve with alleviation of the underlying condition. Dyshematopoiesis had been reported in association with the following acquired conditions in dogs and cats:[155,156,158]

(1) Toxicants: lead toxicity, chemotherapeutic drugs (e.g., vincristine, antimetabolites, alkylating agents, folate inhibitors), estrogen, cephalosporins, chloramphenicol, phenobarbital, and colchicine
(2) Nutritional deficiency: Fe deficiency
(3) Immune-mediated disease: IMHA (dyserythropoiesis) and immune-mediated thrombocytopenia (dysmegakaryopoiesis)
(4) Inflammation (sideroblastic anemias): septicemia, pyometra, pancreatitis, hepatitis, glomerulonephritis, and feline infectious peritonitis
(5) Neoplasms: lymphoma, multiple myeloma
(6) Myelofibrosis, although dyshematopoiesis associated with myelofibrosis might be neoplastic in some cases

4. *Myelodysplastic/myeloproliferative neoplasms* (MDS/MPN) (Table 6.5) include conditions that may have myelodysplastic and myeloproliferative features.[159]

a. *Chronic myelomonocytic leukemia* fulfills the criteria and occurs in dogs. There is typically leukopenia and nonregenerative anemia with a hypercellular bone marrow and atypical hypersegmented monocytoid cells in blood, bone marrow, and often lymph nodes. Fewer than 20 % of the nucleated cells are blasts, and the disorder has a chronic course.
b. *Atypical chronic myeloid leukemia* (also called *MDS/MPN with neutrophilia*) was diagnosed in a dog with neutrophilic leukocytosis and a left shift (but no monocytosis), anemia and thrombocytopenia, trilineage dysplasia, < 20 % blasts in the bone marrow, and an insufficient percentage of cells with a BCR-ABL translocation to support CML.[160]
c. Other myelodysplastic/myeloproliferative neoplasms described in human patients have not been recognized in domestic mammals.

5. *Myeloproliferative neoplasms* (MPNs): This group includes several specific chronic clonal disorders of pluripotential stem cells characterized by excessive proliferation or accumulation of differentiated neoplastic cells that have few or no microscopic or functional abnormalities (relatively normal maturation) (Table 6.5). Tools to detect clonality and prove the presence of neoplasia have limited availability for these conditions in domestic mammals. Diagnosis is often based on exclusion of other conditions, but differentiation from nonneoplastic conditions is difficult because the cells are mature and well differentiated (Figure 6.8).
   a. *Chronic myeloid leukemia* (*chronic myelogenous leukemia, chronic granulocytic leukemia*) (CML) is characterized by persistent neutrophilia and typically a left shift, often with eosinophilia and basophilia. The neutrophilia can become marked and may progress to a terminal blast crisis. The diagnostic hallmark in people is the presence of a reciprocal chromosomal translocation producing a breakpoint cluster region-Abelson murine leukemia (BCR-ABL) fusion gene on the so-called Philadelphia chromosome (the Raleigh chromosome in dogs). This gene causes production of a protein with constitutive tyrosine kinase activity leading to altered apoptotic and cell division pathways that promote cell proliferation and accumulation. The translocation is not specific for chronic myeloid leukemia. It has been reported in dogs with chronic myeloid leukemia,[161] chronic myelomonocytic leukemia,[162] acute myeloid leukemia,[163] and suspected chronic monocytic leukemia.[164]
   b. *Chronic neutrophilic leukemia* is a sustained clonal expansion of only neutrophils, primarily mature and lacking significant dysplasia, with < 1 % circulating blasts and < 5 % bone marrow myeloblasts. Toxic granulation and Döhle bodies may be present. In people, it is associated with mutations of the gene for the G-CSF receptor. It must be differentiated from other causes of neoplastic or nonneoplastic neutrophilia, which, without genetic testing, may not be possible.
   c. *Chronic eosinophilic leukemia* is an otherwise unexplained eosinophilia with (i) a known related clonal cytogenetic abnormality (not yet determined in domestic species) or (ii) either > 2 % blasts in the blood or 5–19 % blasts in the bone marrow.
   d. *Polycythemia vera* is characterized by persistent and progressive erythrocytosis that must be differentiated from other causes of erythrocytosis (see Chapter 3). There is an associated progressive increase in erythropoiesis, granulopoiesis, and megakaryopoiesis that is considered a major criterion for the diagnosis and can be evaluated by bone marrow biopsy. A *JAK2* gain-of-function mutation is also a major diagnostic criterion in people, and a *JAK2* mutation has been reported in an erythrocytotic dog.[165] JAK2 (Janus kinase 2) is a tyrosine kinase involved in cytokine receptor signaling; mutations can cause constitutive signaling that promotes proliferation without increased Epo, thrombopoietin, or G-CSF. Decreased serum or plasma Epo concentration is also supportive if a validated species-specific assay with low enough detection limits is available.
   e. *Essential thrombocythemia* is characterized by persistent thrombocytosis and selectively increased mature megakaryocytes that may be large and hyperlobulated. The diagnosis is generally reached by excluding causes of reactive thrombocytosis (see Chapter 4) and other myeloid neoplasms. In people, identification of an associated underlying mutation can be helpful; the most commonly targeted genes are *JAK2, CALR* (codes for calreticulin), and *MPL* (codes for the thrombopoietin receptor).

f. *Primary myelofibrosis* (previously *chronic idiopathic myelofibrosis*) in people is categorized into prefibrotic and overt fibrotic forms. Diagnosis primarily involves demonstrating megakaryocyte proliferation and one of the typical mutations (*JAK2, CALR*, or *MPL*) or other marker of clonality without meeting criteria for other myeloid neoplasms. There is progressive development of reticulin and then collagen myelofibrosis that may be preceded by unexplained anemia, leukocytosis, and/or splenomegaly. An analogous disorder of primary myelofibrosis may occur in domestic mammals, but supportive molecular genetic evidence has not been reported.[123,166,167]

g. *Unclassifiable MPN*: These are MPN-like conditions that do not meet criteria for other MPN categories.

6. *Mast cell neoplasms*: Mastocytemia may be reactive (nonneoplastic) or neoplastic. When neoplastic, it is usually secondary to mast cell neoplasms arising outside the bone marrow (this may be called *secondary mast cell leukemia*). Mastocytemia is rarely caused by *primary mast cell leukemia*, which originates in bone marrow from hematopoietic cells and is characterized by a bone marrow and blood distribution of mast cells; however, secondary infiltrates can develop in other organs.

C. *Lymphoid neoplasms* (Figure 6.8) derive from undifferentiated or differentiated lymphoid cells, including plasma cells. Neoplasms of lymphocytes or lymphoblasts have been classically divided into *lymphomas* (solid tissue origin, see Lymph Node Classifications, IV. Nodal Lymphoid Neoplasms) and *leukemias* (primarily bone marrow and blood origin and distribution). Lymphomas may enter a leukemic phase with bone marrow and blood involvement, but a predominant tissue distribution and late development of blood involvement signal lymphoma. Certain neoplasms are classified as combined "leukemia/lymphoma" because the specific lymphocyte subtype is more important to clinical progression and management than is the distribution pattern. The term *leukosis* is used in cattle for lymphoid neoplasms (lymphoma or leukemia) caused by bovine leukemia virus.

1. Leukemias of lymphocytes may be divided by general cell type into B-lymphocyte, T-lymphocyte, or rarely NK-lymphocyte leukemias (Table 6.5). Immunophenotyping and cytochemical analysis is often required to determine if immature cells are myeloid or lymphoid, as well as to identify specific cell subtypes (see the following Section E.3 and 4). There are acute and chronic forms of leukemia for each general cell type (B, T, and NK), but it is not always clear microscopically if leukemic lymphocytes have immature features of an acute leukemia or mature features of a chronic leukemia (or if cells with immature features are lymphoid or myeloid). Fine to coarse, reddish cytoplasmic granulation of mature lymphocytes indicates that they are T lymphocytes or rarely NK lymphocytes.
2. The WHO currently includes the following categories for human lymphoid leukemias:[168]
   a. Immature (precursor): (i) B-lymphoblastic leukemia/lymphoma and (ii) T-lymphoblastic leukemia/lymphoma
   b. Mature: (i) chronic B-lymphocytic leukemia/small lymphocytic lymphoma, (ii) splenic B-cell leukemias, and (iii) mature T-cell and NK-cell leukemias, including T-prolymphocytic leukemia, T-large granular lymphocytic leukemia, NK-large granular lymphocytic leukemia, adult T-cell leukemia/lymphoma, Sezary syndrome, and aggressive NK-cell leukemia
   c. These are differentiated based on clinical, morphologic, and genetic/molecular data, much of which is limited in veterinary species.

3. Lymphoid leukemias in veterinary species are commonly characterized based on the morphologic or immunophenotypic (CD34+) immaturity of the population, the general lymphocyte type (B, T, rarely NK), the presence or absence of cytoplasmic granules, and clinical findings. Increasing use of immunophenotyping is allowing further characterization and prognostic information, particularly for dogs. Lymphoid leukemias are most commonly reported in dogs and cats; relatively few have been reported in horses, cattle, or camelids.
   a. *Chronic B-lymphocytic leukemias*:[169–172] Specific diagnostic criteria are not available for domestic mammals, but these leukemias are generally characterized by persistent and otherwise unexplained B lymphocytosis of mostly a monotypic population of mature (CD34–) small lymphocytes with scant cytoplasm and nuclei with clumped chromatin and no apparent nucleoli. Blood lymphocytosis can be extreme, and moderate (> 30 %) to marked bone marrow infiltration by the same population is also present. Persistent, progressive lymphocytosis and exclusion of nonneoplastic causes of lymphocytosis are important for reaching the specific diagnosis, as is exclusion of leukemic extension from certain lymphomas (e.g., mantle cell, marginal zone, or follicular type in dogs). An immunoglobulin gene rearrangement is typically present (PARR). Splenomegaly and lymphadenomegaly may occur, as can anemia, thrombocytopenia, and monoclonal gammopathies. The disorder usually affects older animals and is generally indolent and often detected in asymptomatic patients; however, subsets with varied behavior may exist, and a small percentage of cases evolve into aggressive lymphoma.[173]
   b. *Chronic T-lymphocytic leukemias*:[169,171172,174] Specific diagnostic criteria are not available for domestic mammals, but these leukemias are generally characterized by persistent and otherwise unexplained T lymphocytosis of mostly a monotypic population of mature (CD34–) lymphocytes with clumped chromatin, few or no apparent nucleoli, and often relatively large amounts (for lymphocytes) of pale cytoplasm, making them overall intermediate in size despite relatively small nuclei. None to most of the cells are granular lymphocytes with fine to coarse reddish cytoplasmic granules, often in a cluster; in dogs, these granular lymphocyte leukemias, often called large granular lymphocyte (LGL) leukemias, arise in the splenic red pulp, not the bone marrow, and are relatively common. Other leukemic T cells with mature nuclear features and pale cytoplasm are seen with T-zone lymphoma/leukemia, a disorder that is indolent (with or without the leukemic state) and typically has a CD45– phenotype that facilitates diagnosis by flow cytometry (CD45 positivity has been reported with T-zone lymphoma, but CD45 expression is generally expected with T-zone hyperplasia and not with T-zone neoplasia).[175] Persistent, progressive lymphocytosis and exclusion of nonneoplastic causes of lymphocytosis are important to reach a specific diagnosis. Splenomegaly is common, and anemia and/or thrombocytopenia may be present. The disorder usually affects older animals and is indolent, but rare cases may evolve into aggressive lymphoma.[173]
   c. *Acute lymphoid leukemias*:[139,169,176,177] These leukemias include acute T-cell or B-cell lymphoblastic leukemias and leukemias associated with large B-cell or T-cell lymphomas, the latter technically falling into lymphoma categories rather than acute lymphoblastic leukemia. Lymphomas begin outside the bone marrow and primarily affect other organs but may progress to the marrow and blood, making differentiation from a leukemia difficult. The lymphoblastic leukemias are generally

characterized by a monotypic lymphocytosis of intermediate cells (usually CD34+) with dispersed or finely stippled chromatin but inconspicuous nucleoli. Large granular lymphocytes with immature nuclear features support acute granular T-lymphocyte leukemia, likely of splenic origin. When intermediate to large lymphocytes with blue agranular cytoplasm and prominent nucleoli predominate, an underlying aggressive peripheral T-cell lymphoma is likely. In acute lymphoblastic leukemia, over 20 % of the nucleated cells in the blood or bone marrow are by definition the neoplastic population. Blood lymphocytosis can be extreme, and the marrow is typically hypercellular with little fat and with myelophthisic hypoplasia of other lineages. Therefore, cytopenias are common, including neutropenia and thrombocytopenia, the latter often being more severe than in chronic lymphocytic leukemia. Splenomegaly and lymphadenomegaly may be present. Immunophenotyping and possibly cytochemical evaluation are needed to exclude AML and to characterize the cell type. Clonality results cannot be used to definitively determine cell type. Acute lymphoid leukemia has a rapid clinical course. It is uncommon in cats, rarely reported in horses, and occurs in calves and adult cattle, for which bovine leukemia virus infection may play a role.

   d. *Prolymphocytic leukemias*: These leukemias are classified as chronic and consist of mature ("prolymphocyte" is a misnomer) T or B lymphocytes, but they are aggressive compared to other chronic leukemias. In people, diagnosis of these rare leukemias requires consideration of morphologic appearance, immunophenotyping panels, and molecular abnormalities; they have several morphologic variations and immunophenotypic overlap with other lymphoid neoplasms. In dogs, prolymphocytic leukemia has been diagnosed based largely on microscopic cellular features alone: many intermediate cells with irregularly clumped chromatin forming prominent chromocenters separated by euchromatic spaces, and fewer cells with less clumped chromatin and single nucleoli.
   e. *T-cell granular lymphocytic leukemia or lymphoma in the bone marrow of dogs*: In reported cases of this rare disorder, there has been bone marrow proliferation of granular T lymphocytes without blood lymphocytosis or definitive splenic involvement, and it has been associated with cytopenias, particularly neutropenia.[50,51,178] Clonality has been demonstrated by PARR, which should be used to differentiate a neoplasm from precursor-targeted immune-mediated neutropenia associated with inflammatory small granular lymphocytosis in the marrow.

4. Lymphoma originating in any tissue may eventually involve the bone marrow and blood (see Lymph Nodes: Major Concepts and Terms).
5. *Plasma cell myeloma* is a clonal proliferation of differentiated B lymphocytes within the bone marrow, typically at multiple sites, in which case it can be referred to as *multiple myeloma* (Figure 6.11A). Circulating plasma cells are uncommon.
   a. Although the cells are differentiated, they may have varying degrees of maturation and may not have a mature plasma cell appearance.
   b. These neoplasms are often associated with osteolysis and monoclonal gammopathies due to excessive production of immunoglobulin A (IgA), IgG, or IgM. A small minority of plasma cell myelomas are oligosecretory or nonsecretory, so hyperglobulinemia is not present; immunoelectrophoresis or liquid chromatography with tandem mass spectrometry may detect a monoclonal protein (see Chapter 7).

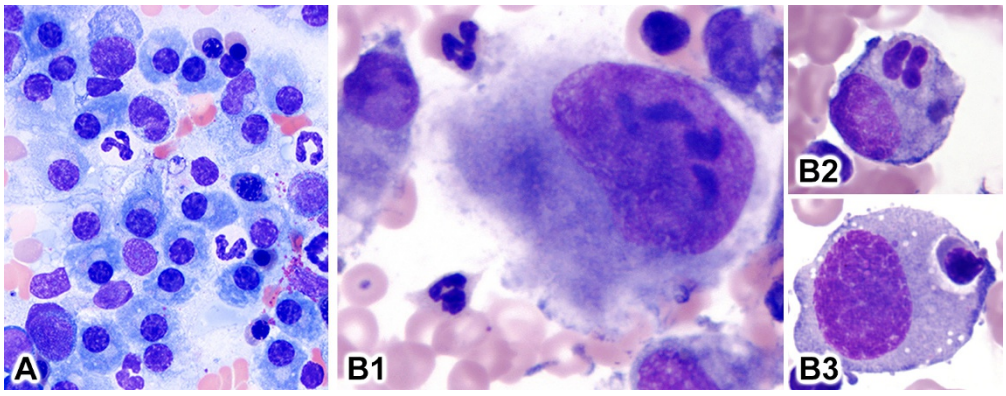


Figure 6.11. A. Plasma cell myeloma, canine bone marrow. Relatively well-differentiated plasma cells effaced much of the marrow and were associated with osteolysis and pancytopenia. B. Histiocytic sarcoma, canine bone marrow. There is marked anisocytosis with a very large cell containing atypical nucleoli (B1) and nonselective phagocytosis of cells including a neutrophil (B2) and a rubricyte (B3).

c. Other laboratory abnormalities may include Bence Jones proteinuria, hypercalcemia, hypoalbuminemia, azotemia, and hyperviscosity.
d. Demonstration of end-organ damage such as otherwise unexplained cytopenias, lytic bone lesions, or renal disease may be required to differentiate plasma cell myeloma from reactive plasmacytosis, particularly when the cells lack atypia.

6. A cutaneous or noncutaneous proliferation of neoplastic plasma cells outside of the bone marrow is an *extramedullary plasmacytoma*.
7. Plasma cell leukemia is very rare but may occur secondary to a plasma cell myeloma or as a primary leukemic proliferation.[179,180]

D. *Histiocytic neoplasms*:[181] Histiocytic malignancies consist of cells that are derived from monocyte or dendritic cell precursors, but they do not typically begin in the bone marrow. Dendritic cells are specialized for antigen presentation, and macrophages are more specialized for phagocytosis. The functions of these two cell types are reflected by their immunophenotypes, which are used to differentiate them.

1. In dogs, *hemophagocytic histiocytic sarcoma* is a proliferation of splenic red pulp and bone marrow macrophages that express, among other markers, CD11d/CD18 and MHCII but lack expression of CD11c and have weak or no expression of CD1a.[181] Rare cases in cats are also thought to originate from macrophages, but immunophenotypic results are incomplete.
   a. Neoplastic cells in the bone marrow cells may appear relatively well differentiated or atypical (Figure 6.11B). Neoplastic cells are consistently present in the spleen, where they may have more atypia. Other tissues may be involved (e.g., liver and lung).
   b. Most dogs have a regenerative anemia, thrombocytopenia, hypoalbuminemia, and hypocholesterolemia, a pattern that is not specific for hemophagocytic histiocytic sarcoma. Bone marrow is commonly hypercellular with neoplastic infiltrates. Erythroid hyperplasia is often present in response to RBC destruction, and myeloid and megakaryocytic hyperplasia may be present secondary to hemophagia or inflammation. Splenic hematopoiesis can be increased and accompanied by splenic histiocytic infiltrates.

2. *Histiocytic sarcoma* (previously called *malignant histiocytosis*) of dendritic cell origin is a localized or disseminated malignant proliferation of interstitial dendritic cells which express, among other markers, CD1a (CD1), MHCII, and CD11c/CD18 but not CD11d.[181,182]
    a. Pleomorphic populations of neoplastic cells may be present in the bone marrow, spleen, lungs, dermis/subcutis, lymph nodes, liver, and periarticular tissues. Phagocytosis may be present but is not as pronounced as with hemophagocytic histiocytic sarcoma.
    b. Other findings are variable and nonspecific, including anorexia, lethargy, weakness, and weight loss. Any breed may be affected, but predisposed breeds include Bernese mountain dogs, rottweilers, golden retrievers, Labrador retrievers, and flat-coated retrievers.
3. When these malignancies affect the marrow, they must be differentiated from (i) systemic (reactive) histiocytosis, a nonneoplastic proliferation of activated dermal interstitial dendritic cells and T lymphocytes that may undergo spontaneous remission and that rarely progresses to a malignancy, (ii) nonneoplastic hemophagocytic syndromes occurring in association with inflammatory or neoplastic disease, (iii) immune-mediated destruction of hematopoietic cells, and (iv) macrophagic inflammation.
    a. Malignant histiocytic proliferations typically have atypia (Figure 6.11B), though it may be more apparent in other tissues. The number of cells in the bone marrow is typically greater than in other conditions that comprise the differential diagnosis, and cells are more likely to be in clusters.
    b. Phagocytic activity typically appears nonselective in hemophagocytic histiocytic sarcoma and hemophagocytic syndrome (Figure 6.11B) but is typically selective for particular lineages and maturation stages in precursor-targeted immune-mediated anemia and neutropenia.
    c. With inflammation, other inflammatory cells may be present, as may organisms.
    d. Attempts have been made using flow cytometry to differentiate malignant and benign histiocytic bone marrow proliferations.[183] Proliferating histiocytes in reactive histiocytosis are CD4+, whereas they are CD4– in histiocytic malignancies.

E. Methods of classifying hemic cell neoplasms (Figure 6.8)
1. The major goal of classification schemes is to determine and communicate the cell of origin with hopes that it can be used to reliably provide prognostic information and therapeutic recommendations. The most reliable classification comes with a multifaceted approach including (i) clinical information, (ii) microscopic evaluation of blood, bone marrow, and other tissues by experienced hematologists, (iii) cytochemical testing (acute leukemias), (iv) broad immunophenotyping panels, (v) sometimes electron microscopy, and, as they are developed and shown to be useful, (vi) molecular and cytogenetic tests. However, a hemic neoplasm may not be classifiable given current knowledge, diagnostic limitations, and the biological variation of cancer.
2. Microscopic evaluation of Wright-stained blood films or bone marrow preparations
    a. The primary value of these evaluations is to detect the presence of neoplastic hemic cells. This may be obvious when there is a large population of atypical cells, but it may be difficult when there are fewer cells or when neoplastic cells have mature features compatible with an inflammatory population.
    b. A first step in characterizing a neoplasm is to determine if the cells have nuclear or cytoplasmic features that are expected in certain differentiated cell lines.[184]

When the cells are immature or lack differentiation, it can be very difficult to classify the cells reliably by their light microscopic appearance; undifferentiated cells could be of one of several cell types.

c. Most neoplasms are classified most accurately by other methods after first being identified as neoplasms by microscopic inspection.

3. Cytochemical reactions[129,176,185–187]
   a. These are helpful for differentiating the lineage(s) of leukemic cells.
   b. The basic premise behind the use of cytochemistry is that leukemic cells may contain enzymes or compounds that occur in or are unique to certain cell lines. For example, neutrophils, canine eosinophils, and monocytes contain MPO, whereas lymphocytes, RBC precursors, and megakaryocytes do not. However, poorly differentiated myeloid precursors may not contain enough analyte for a positive reaction, and reactions may be equivocal.
   c. A cell's enzymes are detected by reactions that form colored precipitates which label the cell at sites of enzymatic activity. Certain nonenzymatic targets such as phospholipids are detected directly by staining.
   d. Reactions may be more prominent in immature cells than in mature cells, or vice versa. For example, leukocyte alkaline phosphatase activity is present in immature canine cells but not mature neutrophils or monocytes; the positive immature cells have been considered myeloblasts,[186–189] but evidence suggests that they are monoblasts.[176] Positive results for cells in canine leukemias thought to be granulocytic may instead have reflected monocytic components of myelomonocytic leukemias, and cells interpreted as myelocytes from healthy dogs may have been monocytoid as well.
   e. The use of cytochemistry for differentiation of leukemias is limited to a few special hematology laboratories; it is of most value when a panel is used (Table 6.6) and when assessed in conjunction with other findings. Cells can be assessed for contents not shown in Table 6.6, e.g., acid phosphatase or material positive for periodic acid Schiff staining.
   f. Positive labeling and staining of acute leukemia cells for MPO, phospholipid, chloroacetate esterase, leukocyte alkaline phosphatase, or α-naphthyl butyrate esterase (diffuse) supports a nonlymphoid lineage. Labeling with α-naphthyl butyrate esterase (diffuse) and chloroacetate esterase supports a myelomonocytic population with combined granulocyte (myelo) and monocytic differentiation. Cells that appear lymphoid in Wright-stained marrow or blood often prove to be myeloid/monocytic when cytochemistry is applied.
   g. Some cytochemical targets can be detected in appropriately fixed plastic-embedded tissues.[129]

4. *Immunophenotyping*[176,190] is the use of antibodies to detect cellular epitopes that help to classify cell types. It is typically used to characterize neoplasms for diagnostic and prognostic purposes, not to confirm the presence of a neoplasm. Immunophenotyping is limited by availability of species-specific or species-validated antibodies to epitopes of interest, and interpretation is limited in those species for which there are fewer validated antibodies and less knowledge of hematologic neoplasms and their expected characteristics.
   a. The use of monoclonal antibodies has enabled the identification of antigens on or in a variety of cell types. If a group or cluster of monoclonal antibodies recognizes the same antigen, that antigen is assigned a CD number (Table 6.7).

Table 6.6. Expected cytochemical reactions of normal or neoplastic hemic cells in dogs and cats

	Cytochemical target[a]				
Cell	MPO	SBB	CAE	LALP	αNBE
Neutrophil[b]	+	+	+	–[c]	–
Lymphocyte	–	–	–	±[d]	–/focal +[e]
Monocyte	±	±	–	+[c] (immature)	+ (diffuse)
Eosinophil	+ in dog – in cat	+ in dog – in cat	±	+	–
Basophil	–	–	+	±	–
Megakaryocyte	–	–	±	–	±

[a] MPO, myeloperoxidase; SBB, Sudan black B; CAE, chloroacetate esterase; LALP, leukocyte alkaline phosphatase; αNBE, α-naphthyl butyrate esterase

[b] Reactions are similar in neutrophil precursors at least back through progranulocytes and likely through myeloblasts.

[c] Historically, LALP positivity was reported to occur in some immature canine neutrophils (granulocyte precursors). In contrast, LALP was more recently reported to be positive in canine monoblasts rather than early granulocytes; this was based on alignment with other monocytic markers in leukemic cells and cells with features suggestive of promonocytes.[176] Considering LALP a monocyte marker rather than a myeloid marker changes the classification of some leukemias from myelomonocytic to monocytic.

[d] Reported LALP results for lymphocytes vary, but positivity has been reported for some B lymphocytes in mantle zones and follicular centers, and for some T lymphocytes.

[e] Some T lymphocytes have focal positive cytoplasmic reactions for αNBE.

Note: Negative results for neoplastic cells do not exclude cell lines; positive results are supportive, and ± indicates weak or sporadic positive results reported in nonneoplastic or neoplastic cells.

b. The CD antigens are best characterized on human and mouse cells, but many of the same CD antigens are found on cells of domestic mammals.

c. Cells from blood, bone marrow, lymph node, cavitary fluids, or other tissues may be immunophenotyped by (i) flow cytometry, (ii) immunocytochemical labeling of air-dried preparations, or (iii) immunohistochemical labeling of fixed or frozen histologic sections. Some antibodies can be used on unfixed tissue but not on fixed tissue, some antibodies are not available with fluorochromes for flow cytometry, and some targets are intracellular (cytoplasmic or nuclear) and not assessable with flow cytometric methods for surface antigens. Full characterization of a cell population may require multiple methods.

d. An antibody used for immunophenotyping allows visualization of positive cells by tagging them directly or indirectly with a fluorescent molecule or a colored precipitate that forms at the site of binding. This is called *immunolabeling*, the label being attached to the target epitope. The term *immunostaining* is also commonly used, but no dye binds or penetrates the target molecules as occurs with staining.

e. Immunophenotyping should be done with panels of antibodies to detect constellations of antigen expression that reflect the cell lineage and stage of maturation. Individual antigen reactivity may be misleading because most immunophenotyping antibodies react with cells of multiple lineages, and neoplastic cells may have aberrant antigen expression, either expressing antigens that their nonneoplastic counterparts do not express or having reduced expression of antigens that their nonneoplastic counterparts do express.

Table 6.7. Selected immunophenotyping targets (antigens) useful in immunodiagnostics of hemic cells in dogs

Cell target	Function	Cells detected	Comments
BLA.36	Surface glycoprotein; may help regulate proliferation	B lymphocytes, plasma cells; possibly some DCs	B-lineage marker
CD1a–c	Antigen-presenting molecules	Cortical thymocytes, DCs; CD1c also expressed on some macrophages and B lymphocytes	Detects DCs; not expressed by mature T lymphocytes but may be by some T-cell lymphoblastic neoplasms
CD3 (CD3ε)	Part of the TCR complex	Mature T lymphocytes (possibly activated NK lymphocytes)	Identifies αβ- and γδ-T lymphocyte leukemia/lymphoma
CD4	Protein that associates with the TCR	MHC class II–restricted helper T cells, neutrophils (only dogs)	Subtypes T cells; monocytes, macrophages, and DCs can express when activated
CD5	Negative regulator of TCR signaling	Mature T lymphocytes; NK lymphocytes (weak)	T-lineage marker; B cells may express it
CD8 (α or β)	Dimeric protein associated with TCR; binds class I MHC during antigen-specific activation	MHC class I–restricted cytotoxic T lymphocytes (usually CD8αβ); subset of NK cells (CD8αα)	Helps subtype T lymphocytes
CD11a–d	α-Subunit of the WBC adhesion molecule family of β_2-integrins	CD11a: all WBCs	Panleukocyte marker
		CD11b: granulocytes, monocytes; some macrophages and NK cells	Marker of nonlymphoid WBCs
		CD11c: granulocytes, monocytes, DCs	Helps identify histiocytic neoplasms of antigen-presenting cells
		CD11d: macrophages and γδ-T cells from splenic red pulp, some granular lymphocytes	Helps identify hemophagocytic histiocytic sarcoma, hepatosplenic T-cell lymphoma, some T-cell leukemias
CD14	Co-receptor for bacterial lipopolysaccharide	Monocytes; some macrophages and DCs	Monocyte marker
CD18	β_2-Subunit of the WBC adhesion molecule family of β_2-integrins	All WBCs (includes macrophages, DCs); lymphocytes express less (antibodies or fixation may affect detection)	Supports hemic cell origin; deficient in dogs with leukocyte adhesion deficiency
CD20	Enables optimal B-lymphocyte response to antigens	B lymphocytes not in earliest stage of development; not plasma cells	B-lineage marker (not all B cells express it); labels some T-cell lymphoma cells or B cells may not express

Continues

Table 6.7. Selected immunophenotyping targets (antigens) useful in immunodiagnostics of hemic cells in dogs (Continued)

Cell target	Function	Cells detected	Comments
CD21, CD21-like	Complement receptor (CR2); linked to BCR complex	Mature B lymphocytes, follicular DCs (± plasma cells)	B-lineage marker
CD22	Inhibits B cell responses; promotes self-tolerance	B lymphocytes	B-lineage marker; limited information on spectrum of B-lymphocyte labeling
CD34	Surface glycoprotein that enhances cell migration	Hematopoietic stem cells and progenitor cells; endothelial cells and some other nonhemic cells	Supports acute form of myeloid or lymphoid leukemia, but some B-cell lymphomas express CD34
CD41	α_2-Integrin of the fibrinogen receptor	Megakaryocytes (and platelets)	Megakaryocyte lineage marker; see CD61
CD45	Membrane tyrosine phosphatase; regulates many cell processes, e.g., mitosis and differentiation	All nonerythroid hemic cells; CD45RA isoform is on B cells and some T cells and monocytes	Panleukocyte marker; T-zone lymphoma cells are typically negative; immature B cell neoplasms may express little or none
CD61	β_3-Integrin of the fibrinogen receptor	Megakaryocytes, platelets; other cells include endothelial cells	Megakaryocyte lineage marker; other cells may label if platelets adhere to them
CD71	Transferrin receptor 1	Nucleated erythroid cells; reticulocytes (flow cytometry)	Erythroid-lineage marker; helpful to support erythroleukemia or early-stage PIMA
CD79a	Part of the BCR complex	B lymphocytes, plasma cells, megakaryocytes	B-cell lineage marker, but expressed by some myeloid and T cells
CD90 (Thy-1)	Surface glycoprotein involved in cell adhesion and signaling	T and some B cells, thymocytes, monocytes, some DCs, stem cells	Lymphocyte marker mostly; canine eosinophils may express it
MHCII	Involved in antigen presentation to helper T cells	B lymphocytes, macrophages, most DCs and monocytes, nonhemic cells	Supports antigen-presenting cells; activated or neoplastic T cells may express it
MPO	Myeloperoxidase	Granulocytic and monocytic cells	Granulocyte and monocyte marker
MUM1 (IRF1)	Transcription factor for B, T, and plasma cell differentiation	Plasma cells, some B lymphocytes; DCs, macrophages	Helps identify plasma cells; T cells may aberrantly express MUM1
Pax-5	Transcription factor important in B cell development	B lymphocytes	B-lineage marker (human *PAX5* mutations can cause acute B-cell leukemia without Pax-5 expression)
VWF	von Willebrand factor	Megakaryocytes, platelets	Megakaryocyte lineage marker (weak)

Note: The following can be detected in formalin-fixed tissues with appropriate antigen retrieval and available antibodies: CD3ε, CD5, CD11d, CD18, CD20, CD21-like, CD45, CD45RA, CD61, CD71, CD79a, MHCII, MPO, MUM1, Pax-5, CD90 (Thy-1), and VWF.

ALL, acute lymphocytic leukemia; BCR, B-cell receptor; BLA.36, B lymphocyte antigen, 36 kD; CLL, chronic lymphocytic leukemia; DC, dendritic cell; GP, glycoprotein; IRF4, interferon regulatory protein 4; MHC, major histocompatability complex; MUM1, multiple myeloma oncogene 1; Pax-5, paired box protein 5; Thy-1, thymocyte antigen 1

f. Appropriate immunophenotyping panels differ with the suspected neoplasm, e.g., lymphoma, acute leukemia, histiocytic neoplasm, plasma cell myeloma. A minimal immunophenotyping panel to characterize an acute leukemia, depending on antibody availability for the species of interest, might include a panleukocyte marker (CD18 or CD45), a B-lymphocyte marker (CD20, CD21, CD79a, Pax-5), a T-lymphocyte marker (CD3 or CD3ε), myeloid markers including for dendritic cells (CD11b, MPO, CD1c, MCHII), a megakaryocyte marker (CD61, CD41, von Willebrand factor), and an immaturity marker (CD34). Additional or alternate antibodies can be used to characterize cell types and subtypes.

g. Immunophenotyping results should be interpreted in light of all available historical, clinical, cytologic, histologic, cytochemical, cytogenetic, and molecular genetic findings.

5. Electron microscopy may be useful in some leukemias to provide evidence of a particular cell line; for example, the ultrastructure of leukemic cell cytoplasmic granules may be distinctive for basophil, eosinophil, or mast cell granules. Rarely, immunogold labeling may be used with electron microscopy to detect lineage-selective markers.

6. Molecular and cytogenetic studies may be employed to detect genetic abnormalities that may predict behavior and outcome of some neoplasms. Chromosomal abnormalities in canine leukemia cells have been detected and may aid in the diagnosis and prognosis of certain neoplasms, e.g., the Raleigh (Philadelphia) chromosome in chronic myeloid leukemia.[161] Chromosomal banding studies and molecular genetic techniques are being evaluated. These include fluorescent in situ hybridization, comparative genomic hybridization, and microarrays. Currently, the most-used genetic technique is the species-specific PCR for assessment of lymphocyte clonality, commonly referred to as PARR, an acronym denoting PCR for Antigen Receptor Rearrangements.[177,191–194] Differences in primer set sequences and number used, assay conditions, singleplex versus multiplex testing, sample type (fresh versus fixed), and sample quality and processing can affect analytical or diagnostic performance of PARR assays. Interpretation of PARR results is aided by understanding of the following statements:
   a. With rare exceptions, neoplasms of lymphoid cells are uncontrolled proliferations or accumulations of one clone of lymphocytes.
   b. Antigen-binding regions of B- and T-lymphocyte receptors are encoded by the complementary determining region 3 (CDR3) of the immunoglobulin and TCR genes. The CDR3 region is produced by various recombinations of several genes, generating numerous genetic combinations. Primers are used to amplify conserved regions of these genes, and the products are separated by size. If there is a clonal expansion of cells with one particular CDR3 region detectable with the test primers, separation will yield a dominant band or peak.
   c. PCR assays for B- and T-lymphocyte antigen receptor gene rearrangements are useful to help differentiate lymphoid neoplasms from inflammatory conditions when cytologic or histologic appearance and immunophenotyping are suggestive of lymphoproliferative neoplasms but not definitive for them. They are not needed when there is a definitive microscopic diagnosis, and they should not be done without microscopic evaluation.
      (1) The presence of gene rearrangements supports a neoplasm. B-lymphocyte and T-lymphocyte gene rearrangements usually, but not exclusively, occur in neoplasms of the respective lymphocyte cell types.[192] However, B, T, or both B and T gene rearrangements are common in AML, so PARR results cannot be used to diagnose a lymphoid neoplasm.[140] A poorly differentiated neoplasm with dual PARR clonality may be an AML or a lymphoma.

(2) The absence of a detected gene rearrangement does not exclude a lymphoid neoplasm. Negative results occur in roughly 10–50 % of samples from clonal populations, depending on the assay.[192] Reasons include the following.
  (a) Mutations have altered the clone's DNA sequence to which primers were designed to bind.
  (b) The rearranged genes are not within the target regions of primer sets.
  (c) The chromosome containing the antigen receptor gene has been lost from the clone, or another chromosomal alteration interferes.
  (d) Too few clonal cells are sampled within a polyclonal lymphoid population.
  (e) The clonal cells are precursors (CD34+) that are too immature to have rearranged their antigen receptor genes.
  (f) The cells are not B or T lymphocytes (e.g., NK cells).
  (g) Nontarget DNA was amplified.
  (h) Too little quality DNA could be extracted from the sample (e.g., small sample, damaged DNA).

(3) The assay diagnostic sensitivity varies with the tissue and proportion of lymphocytes that are neoplastic, but clonality can be detected when as little as 0.1–10.0 % of sample DNA is from neoplastic cells. Therefore, it can be used to detect residual disease after treatment or otherwise undetectably small numbers of neoplastic cells during staging. However, false positive clonality may occur with low loads.

(4) Clonal rearrangement of the TCR gene has been detected in canine *Ehrlichia canis* and *Leishmania infantum* infections, although concurrent occult lymphoid neoplasia could not be completely excluded in all cases.[191,195] The detection of clonality by PARR appears to have high specificity for neoplasia.[192]

(5) Small samples may yield discrete bands that appear to be clones but are pseudoclones. Duplicate testing with separate DNA extractions or additional primer sets can be done to decrease the likelihood of misinterpreting pseudoclones as real clones. Detection of clones should be repeatable.

F. Nonhemic neoplasms may be present in bone marrow because of metastasis or extension from adjacent tissue. Types include various carcinomas, melanoma, various soft tissue sarcomas, osteosarcoma and chondrosarcoma, mesothelioma, hepatoblastoma, and nephroblastoma.

INTERPRETING RESULTS OF BONE MARROW EXAMINATIONS

I. Interpretation of the results of bone marrow examinations should address the indication for marrow biopsy by answering the specific question(s) being asked. For example:
  A. Why does an animal have a persistent nonregenerative anemia?
  B. Why does an animal have a persistent neutropenia?
  C. Is there a neoplasm in the bone marrow?

II. Complete interpretation of most bone marrow samples is possible only when there are CBC results for the day the bone marrow was collected.
  A. Hyperplastic and hypoplastic conditions can usually be recognized independently of CBC findings, but the CBC findings help explain the disorder. For example, a hypercellular bone marrow (based on aspirate particle cellularity or histologic assessment of core sections) with an increased M:E ratio indicates there is myeloid hyperplasia (rarely neoplasia) no matter

what the CBC findings are. However, the CBC may reveal a neutropenia indicating there is ineffective neutropoiesis or a marked neutrophilia indicating effective neutropoiesis. The former case suggests immune-mediated neutropenia, whereas the latter case supports an inflammatory process.

B. When marrow cellularity is neither clearly increased nor clearly decreased and there is an abnormal M:E ratio, the CBC provides clues as to the cause of the abnormal ratio.
   1. If the M:E ratio is increased, it may be due to erythroid hypoplasia, neutrophilic (granulocytic, myeloid) hyperplasia, or both. Nonregenerative anemia would support erythroid hypoplasia, whereas neutrophilia and a hematocrit within the reference interval would support neutrophilic (granulocytic, myeloid) hyperplasia.
   2. If the M:E ratio is decreased, it may be due to erythroid hyperplasia, neutrophilic (granulocytic, myeloid) hypoplasia, or both. A regenerative anemia or erythrocytosis would support erythroid hyperplasia, whereas a leukopenia would support neutrophilic (granulocytic, myeloid) hypoplasia.

C. Unless a condition is clearly chronic and stable, it is important that CBC results are collected within 12–24 h of bone marrow collection.
   1. An animal may have a nonregenerative anemia one day but a regenerative anemia the next day (or the reverse).
   2. An animal may be neutropenic one day but neutrophilic the next day (or the reverse).
   3. An animal may be thrombocytopenic one day but thrombocytotic the next day (or the reverse).

D. Examples of the correlation of CBC and bone marrow results for five dogs with various indications for bone marrow evaluation are shown in Figure 6.12. Results must also be interpreted in light of other clinical findings.

LYMPH NODES: MAJOR CONCEPTS AND TERMS

I. Terms and conditions

A. *Lymphadenopathy* is a pathologic state involving a lymph node. In clinical diseases, most lymphadenopathies are recognized by enlarged lymph nodes, i.e., *lymphadenomegaly*. Frequently, lymphadenopathy is used imprecisely to mean enlarged lymph node.

B. The primary reason for a lymph node biopsy is to determine the pathologic process that is causing lymphadenomegaly. Pathologic processes may be present in lymph nodes that are not enlarged, but not as frequently. The following are major disorders or conditions that cause enlarged lymph nodes:
   1. Hyperplasia of lymphoid cells
   2. Inflammation involving lymph nodes
   3. Neoplasms of lymphoid cells
   4. Neoplasms of nonlymphoid cells (typically metastatic neoplasms)

II. Methods

A. Complete descriptions of each phase of a lymph node biopsy (collection, fixation, staining, and examination of lymph node tissue from a living animal) are beyond the scope of this text. Procedures can be found in several cytology sources.[196–198] Major features are provided here.
   1. Sample collection and processing
      a. Typically, lymph node samples are collected from enlarged peripheral lymph nodes: mandibular, prescapular, axillary, popliteal, inguinal, and, occasionally, facial (only

CBC results	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Ref. Int.
Hct (%)	10	10	10	20	10	40–55
Reticulocytes (/µL)	10,000	10,000	10,000	200,000	10,000	10,000–80,000
Neutrophils (/µL)	7000	7000	500	30,000	1000	3000–8000
Platelets (/µL)	300,000	300,000	20,000	10,000	20,000	160,000–400,000
Atypical cells (/µL)	0	0	0	0	Few blasts	none
Bone marrow biopsy results						
Hematopoietic cellularity (%)	20	80	10	85	90	25–75
M:E ratio	3.0	0.6	0.9	0.7	3.9	0.9–1.8

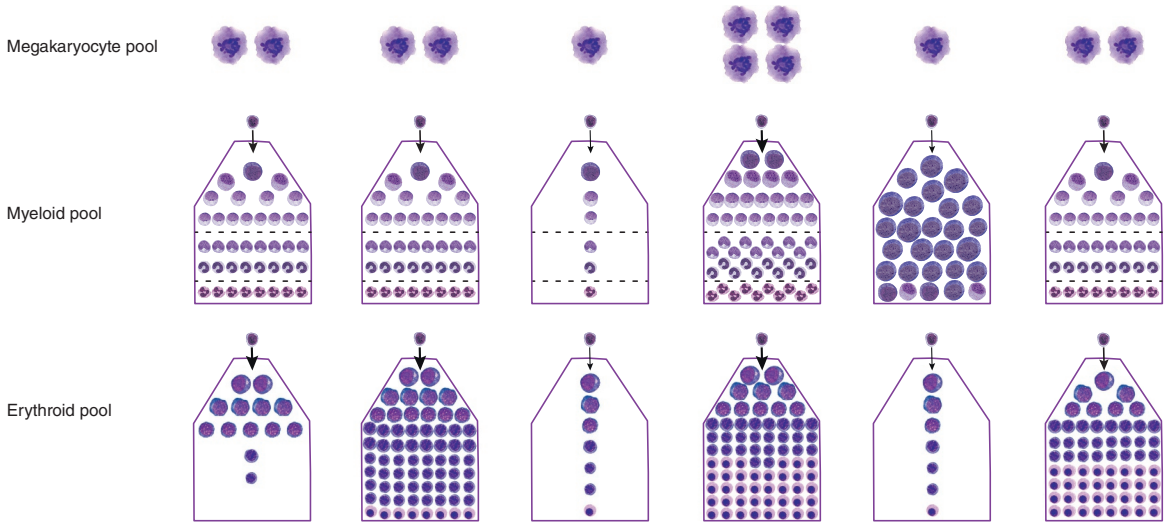


Figure 6.12. Schematic examples of the interpretation of CBC and bone marrow biopsy results. Expected results for healthy dogs are provided in the right column. The number of displayed megakaryocytes reflects the relative marrow megakaryocyte density compared to health. Neutrophil and erythroid pools are represented by diagrams like those in Chapters 2 and 3.

Figure 6.12. *continued*

- Dog 1, unexplained, persistent, nonregenerative, marked anemia: Decreased particle hematopoietic cellularity and an increased M:E ratio indicate erythroid hypocellularity. There appears to be a nearly complete erythroid maturation arrest after the early rubricyte stage. Other lineages are unaffected. This pattern supports PIMA with destruction of rubricytes and hyperplasia of early-stage precursors. Rubriphagocytosis may be present. An absence of erythroid precursors would have supported PRCA.
- Dog 2, unexplained, persistent, nonregenerative, marked anemia: Increased particle hematopoietic cellularity and a decreased M:E ratio indicate erythroid hypercellularity. CBC data indicate it is associated with a nonregenerative anemia; therefore, there is ineffective erythropoiesis. The erythroid lineage is shifted toward immaturity with expansion of all stages except metarubricytes. Other lineages are unaffected. This supports PIMA with targeting of metarubricytes, in which case selective rubriphagocytosis is likely present. Nonselective phagocytosis could suggest hemophagocytic syndrome. MDS would be supported by many dysplastic cells, but the diagnosis requires exclusion of other disorders (e.g., PIMA). Dysplastic cells and ineffective erythropoiesis may also be present with cobalamin deficiency and congenital dyserythropoietic disorders, other causes of ineffective erythropoiesis to consider. Iron-deficiency anemia typically would be apparent from RBC indices (microcytic, hypochromic) and late-stage erythroid precursors would be expanded.
- Dog 3, unexplained, persistent pancytopenia: Decreased particle hematopoietic cellularity, a M:E ratio within the reference interval, and decreased megakaryocytes indicate generalized marrow hypoplasia causing the pancytopenia. If most of the marrow is filled by adipocytes, the findings fit an aplastic anemia (aplastic pancytopenia), which may be caused by infectious, toxic, or immune-mediated mechanisms. If few marrow adipocytes and mostly matrix are present, the findings fit an acute myelosuppressive event such as a toxicity or parvoviral infection. Other findings may suggest other causes of generalized hypoplasia.
- Dog 4, unexplained persistent thrombocytopenia: Increased particle hematopoietic cellularity with a decreased M:E ratio indicates erythroid hypercellularity. The blood reticulocytosis indicates effective erythropoiesis (a regenerative anemia), and thus effective erythroid hyperplasia. This occurs secondary to blood loss or hemolysis. Neutrophilia is evidence for myeloid hyperplasia despite a decreased M:E ratio; the neutrophil lineage is expanded, but the erythroid lineage is expanded more. Myeloid hyperplasia with a neutrophilia is nonspecific evidence of an inflammatory process. Increased megakaryocyte density supports megakaryocytic hyperplasia, an appropriate response to a marked thrombocytopenia caused by decreased platelet survival (consumption or immune-mediated destruction). If evidence of disease that would promote platelet consumption is absent (e.g., no coagulation or fibrinolysis abnormalities, no evidence of vasculitis or neoplasms), immune-mediated thrombocytopenia would be likely, just as it was prior to bone marrow collection. Marrow evaluation has limited value in dogs with severe, unexplained thrombocytopenia.
- Dog 5, pancytopenia with few circulating atypical cells (blasts): Very high particle cellularity and an increased M:E ratio with a predominance of atypical immature myeloid cells supports AML. A decrease in megakaryocytes and erythroid cells suggests myelophthisic megakaryocytic and erythroid hypoplasia; CBC findings of thrombocytopenia and nonregenerative anemia further support this interpretation. Neutropenia is caused by defective neutropoiesis (neoplasia). A leukocytosis of neoplastic cells is common but not always present in AML. When blasts are present in blood or marrow, special tests are often needed to determine if they are myeloid, monocytic, erythroid, megakaryocytic, or lymphoid.

apparent with disease). Internal lymph nodes are also sampled, usually by ultrasound-guided aspiration or during a celiotomy or thoracotomy. When multiple lymph nodes are enlarged, it is wise to sample multiple nodes.

b. Samples may be collected from the lymph node by fine needles (21 g or smaller), Tru-cut needles, wedge incision, or lymph node excision. After the sample is collected, it may be prepared for microscopic analysis, tissue sectioning, culturing, or other procedures.

c. Fine-needle collection is a relatively simple procedure for obtaining cytologic samples, but many errors are made that result in inadequate or suboptimal samples. The following steps may be used as a guide for collecting cytologic samples from superficial lymph nodes or other superficial masses:
   (1) Slides should be made ready.
   (2) Select a 22 gauge needle and a 5–12 mL syringe.
   (3) Prepare the skin as for a venipuncture.
   (4) Isolate and stabilize the lymph node with one hand.
   (5) Insert the needle into the skin and lymph node. A syringe is not necessary at this time, but if using a needle attached to a syringe, moderate suction can be applied after the needle is in the lymph node to help hold cores of tissue in the needle as they are cut by forward jabs. More forceful aspiration to suck material out while leaving the needle in one place can cause significant hemodilution, especially with tissues that are more vascular than lymph nodes.
   (6) Cut a core of tissue with sharp forward cutting motions followed by an optional rotation of the needle to help free the core, withdraw the needle without leaving the node, and repeat up to three times in different directions to obtain a representative sample.
   (7) Withdraw and attach a syringe containing air (relieve suction before withdrawing if using a syringe, then remove the syringe and reattach with air in it).
   (8) Place the needle against a slide, bevel down, and express the sample in a pool. Do not spray small droplets onto the slide.
   (9) Spread the material by one of several techniques to obtain areas on one or more slides that contain thin layers of well-spread intact cells.
      (a) A wedge blood smear technique may be used for thin (more fluid) samples.
      (b) Horizontal or vertical "squash" techniques can be used for thicker samples. A wavy spreading motion can be used with a horizontal technique in order to obtain thin zones of cells.
      (c) Lymph node samples are often very cellular and thick, sometimes too thick for evaluation, so care should be taken to spread material well. If the drop is moderate to large, instead of spreading it all into one thick smear, some of the material can be repeatedly picked up on the underside of the short edge of another slide and spread by a horizontal squash technique onto other slides.
   (10) The air-dried slides should not be fixed (with heat or other fixative) prior to staining or submission, and they should not be exposed to formalin fumes because the gas can severely alter the staining properties of the cells. Keep them clean, covered, and at ambient temperature.

d. To process samples for histologic evaluation, thin slices of excised samples should be placed in fixative for paraffin-embedded sectioning. Neutral-buffered formalin is used most often, but other fixatives as described for bone marrow cores may be used for improved cell detail.

2. Cytologic examination of lymph node samples
   a. This involves the differentiation and characterization of nucleated cells and the identification of organisms or other noncellular structures (e.g., hemosiderin). Thick smears without well flattened cells or with pale staining may limit conclusions (Figure 6.13B and C). Smears with mostly damaged cells are common and suggest traumatic smearing technique, fragile cells, or both.
      (1) The types of lymphocytes are determined by their nuclear diameters, chromatin patterns, the presence and location of nucleoli, and cytoplasmic features. In health, most lymphocytes should be small lymphocytes with clumped chromatin patterns, scant cytoplasm, and inapparent nucleoli.
      (2) Other cells are identified by their unique features. Nonlymphoid cells may include neutrophils, macrophages, eosinophils, mast cells, metastatic neoplastic cells, and hematopoietic precursors.
      (3) Smears consisting of adipocytes and lipid or acinar and ductular epithelial cells with evidence of sample viscosity support sampling of perinodal adipose tissue or a mandibular salivary gland (Figure 6.13D), respectively.

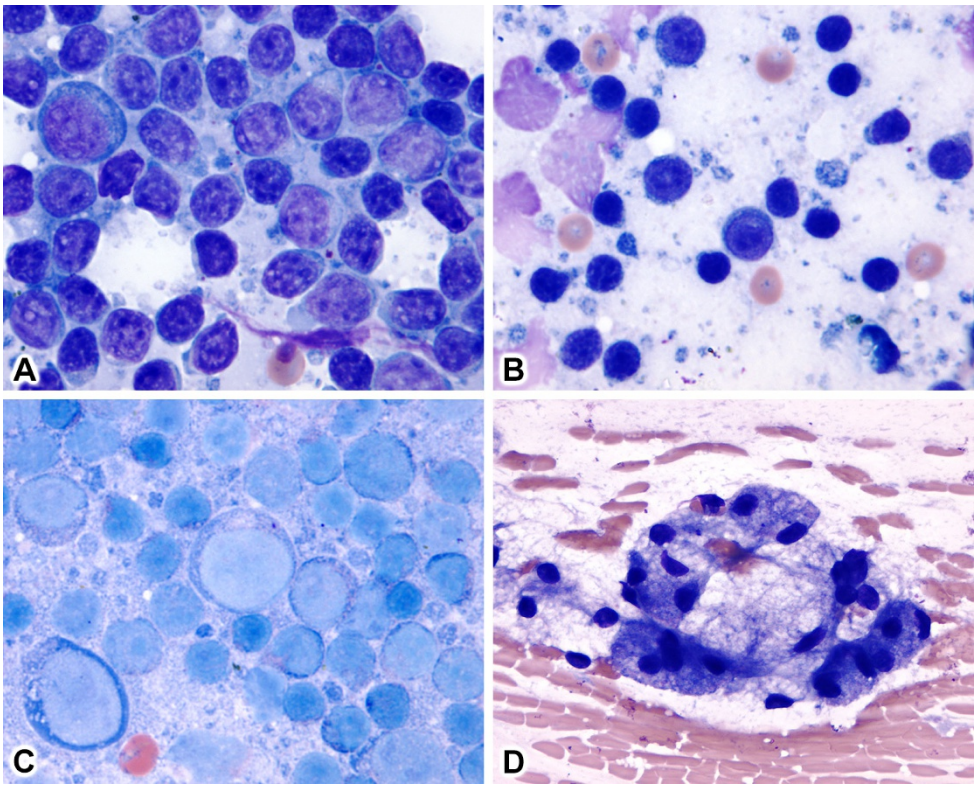


Figure 6.13. A. Canine lymph node smear with predominantly small and intermediate lymphocytes, expected findings in health. B. Thick lymph node smear despite low cell density; lymphocytes are balled up (poached or hard-cooked eggs), thus obscuring cell features and preventing accurate sizing. C. Insufficiently stained lymph node smear; understaining is common because lymphocytes exfoliate well and make thick smears that require more stain. D. Cluster of secretory epithelial cells and rowing of RBCs because of viscous salivary fluid, mandibular salivary gland instead of lymph node, dog.

b. Examinations are best completed on a quality stained sample with a quality microscope by a person who is trained for such examinations.

3. Histologic examination of lymph node tissue, including cell blocks made from aspirated material, is beyond the scope of this textbook. Such examinations should be performed by veterinary pathologists.

B. Cells in lymph nodes of healthy mammals

1. Lymph nodes from healthy mammals are not commonly evaluated. However, microscopists should have a good idea of what should be present in health so that abnormal cell populations or other significant findings can be recognized.
   a. Lymph nodes from healthy animals have heterogeneity caused by the lymphocytes of different sizes and the presence of nonlymphoid cells. Small to intermediate lymphocytes account for the vast majority of the cells (Figure 6.13A). There are low percentages of plasma cells, large lymphocytes, neutrophils, eosinophils, and macrophages.
   b. The expected cell populations in lymph nodes vary with the location of the lymph node, but small lymphocytes always predominate. Mandibular and mesenteric lymph nodes in healthy mammals typically have mildly greater percentages of resident macrophages, plasma cells, neutrophils, and intermediate and large lymphocytes than do other lymph nodes.
2. The basic cytologic classification of lymphocytes is by size: small, intermediate, or large, generally referring to nuclear size. Large granular lymphocytes have a greater proportion of cytoplasm than other types of lymphocytes, so their cell size is relatively large despite nuclei that are not. The size of lymphocytes should be judged where cells are well spread (diameters are maximized). This cannot be emphasized enough. Such regions may not exist on a slide (Figure 6.13B), or they may be present only on certain margins of the smear. Lymphocytes can be measured with a micrometer or compared to "cellular micrometers" such as RBCs, neutrophils, or plasma cell nuclei.
   a. If comparing to RBCs, it is important that the RBC micrometer is singlet and not a doublet. If one is in the appropriate area of a smear, there should be no rouleaux. If comparing to a neutrophil, one must be sure that the neutrophil is well spread like a fried egg and has maximal cytoplasmic area. If it does not, it may be balled up like a poached egg and have a diameter as low as that of a RBC. If comparing to a plasma cell, one must make sure it is a mature plasma cell with a small dense nucleus, not a plasmablast, reactive lymphocyte, or other cell type with an eccentric nucleus and blue cytoplasm.
   b. Cell "micrometer" guidance: In the optimal areas of air-dried cytologic smears, the typical canine RBC diameter is about 7 μm, the canine neutrophil diameter is 12–14 μm, and mature plasma cell nuclei have diameters similar to those of RBCs. RBCs in cats, horses, and ruminants are smaller, so use of canine-based sizing guidelines would be misleading, and classification must be modified accordingly.
   c. Sizing guidelines and implementation vary considerably.
      (1) One system classifies canine lymphocytes by nuclear diameter relative to canine RBC diameters; there are gaps between categories:[196]
         (a) Small: 1–1.5 × a RBC diameter
         (b) Intermediate: 2–2.5 × a RBC diameter
         (c) Large: > 3 × a RBC diameter
      (2) Others have used numeric diameters for classification:[199]
         (a) Small: < 10 μm
         (b) Intermediate: 10–15 μm
         (c) Large: > 15 μm

d. Histologic evaluation: The diameter of a RBC can be used as a size comparison or "micrometer" in formalin-fixed histologic sections as well, but it is substantially less (e.g., 5 µm versus 7 µm, dogs) than in air-dried smears, as are other cell diameters because of cell shrinkage and lack of cell flattening. The RBC used as a histologic "micrometer" should have been sectioned through its greatest diameter.

3. Large lymphocytes with basophilic cytoplasm, finely stippled chromatin patterns, and nucleoli have often been referred to as *lymphoblasts* as is consistent with (i) the terms myeloblast, monoblast, and rubriblast, (ii) unidentified blasts reported as part of a blood WBC differential count, and (iii) lymphocyte blast transformation or mixed lymphocyte reaction assays. However, these cells do not fit the definition of a blast as a developmentally immature precursor cell, so many pathologists use the term "large lymphocyte" instead. In the context of lymphoma/leukemia subtypes, lymphoblasts are relatively small precursor lymphocytes with immature chromatin, no conspicuous nucleoli, and a high proliferation potential.

LYMPH NODE CLASSIFICATIONS

I. Hyperplastic lymph node
   A. *Lymph node hyperplasia* is characterized by increased numbers of B lymphocytes, T lymphocytes, or both. The proportions of different types and sizes of lymphocytes may be the same as in health, in which case hyperplasia is suggested by lymphadenomegaly. There may be increases in large lymphocytes and/or plasma cells, in which case the terms *reactive* or *reactive hyperplasia* are often used in place of hyperplasia, though the nodes are enlarged because of hyperplasia.
   B. A variety of infectious and noninfectious diseases, including bacterial, viral, fungal, and neoplastic disorders, can lead to the stimulation and proliferation of lymphocytes. If there is generalized lymph node hyperplasia, a systemic illness should be considered. If only one node is hyperplastic, a disease within the drainage field of that node should be considered.
   C. There may or may not be a concurrent inflammatory lymphocytosis in mammals with hyperplastic lymph nodes.

II. Reactive lymph node
   A. A node classified as *reactive* typically has increased numbers of plasma cells and/or intermediate or large lymphocytes (Figure 6.14A and B). The percentage of large lymphocytes is expected to be < 50 % in a reactive node and is usually < 10 %. An increase in plasma cells indicates B-lymphocyte stimulation.
   B. Mott cells may be present in reactive nodes where they suggest chronic antigenic stimulation (inflammation). *Mott cells* are plasma cells that are typically packed with round to irregular inclusions of protein called Russell bodies (Figure 6.14A).[200,201] Russell bodies are generally thought to be vesicular structures derived from endoplasmic reticulum and containing mostly insoluble immunoglobulin aggregates. Protein accumulation may occur from cellular indigestion or constipation, an inability of disposal or secretion to keep up with creation.[202] Mott cells may be reactive or neoplastic plasma cells; abnormal immunoglobulin or cell functions in neoplastic cells may promote Russell body formation.
   C. The causes of a reactive lymph node are essentially the same as those for lymph node hyperplasia.

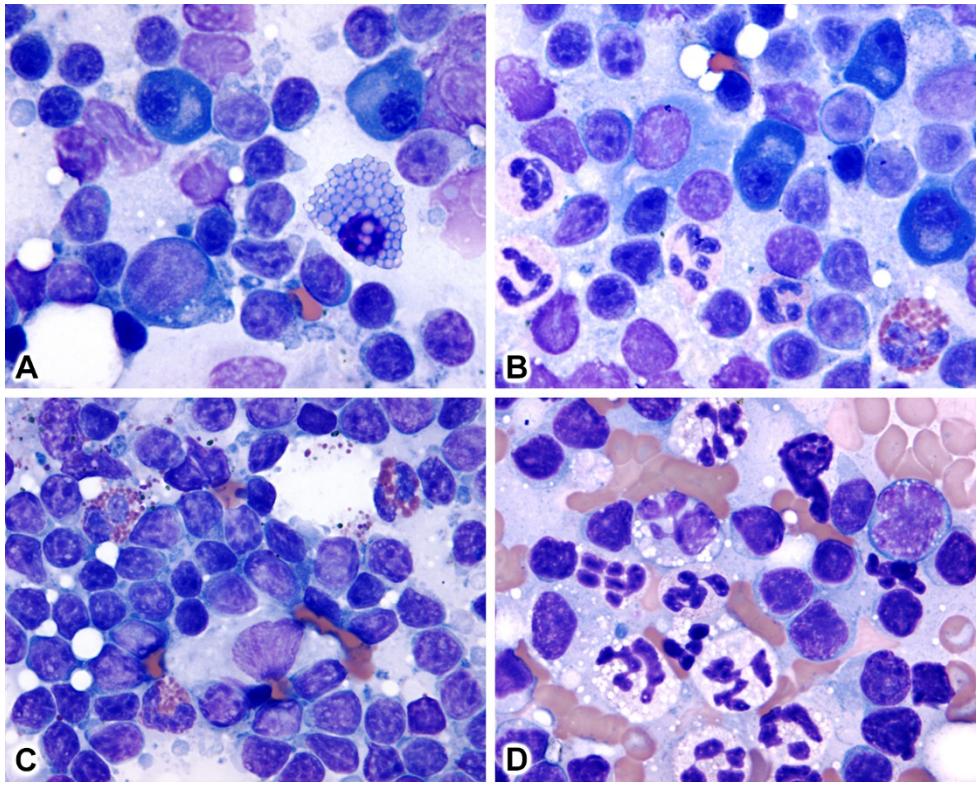


Figure 6.14. A. Three plasma cells including one Mott cell, one large lymphocyte, and small lymphocytes in a reactive lymph node, dog. B. Plasma cells, neutrophils, an eosinophil, and small lymphocytes, reactive lymphadenitis, dog. C. Three eosinophils and mostly small lymphocytes, eosinophilic lymphadenitis, dog. D. Vacuolated neutrophils, a reactive intermediate lymphocyte (*upper right*), and small lymphocytes, neutrophilic lymphadenitis, dog.

III. Lymphadenitis or inflammation in a node's drainage field
   A. *Lymphadenitis* is characterized by an increased number of nonlymphoid inflammatory cells in a lymph node. One inflammatory cell type might dominate (e.g., neutrophils), or there can be a mixture of inflammatory cells (e.g., neutrophils, macrophages, and eosinophils) (Figure 6.14B and D and Figure 6.15).
   B. The cause of the inflammatory state and primary site of inflammation may be within the lymph node or, more commonly, in the node's drainage field. For example, allergic dermatitis may lead to eosinophilic lymphadenitis, or a lymph node draining a necrotic hemorrhagic lesion may contain many macrophages with intracytoplasmic hemosiderin and degraded cellular material.
   C. There may or may not be a concurrent inflammatory leukocytosis.
   D. Organisms such as pyogenic bacteria, *Leishmania* (Figure 6.15A), *Mycobacterium* (Figure 6.15B), *Histoplasma* (Figure 6.15C), *Blastomyces* (Figure 6.15D), *Trypanosoma*, *Prototheca*, *Neorickettsia*, *Cytauxzoon*, and *Toxoplasma* may be present. *Mycoplasma* spp., *Babesia* spp., *Ehrlichia* spp, *Anaplasma* spp., and distemper inclusions may also be seen on (*Mycoplasma*) or within hemic cells.

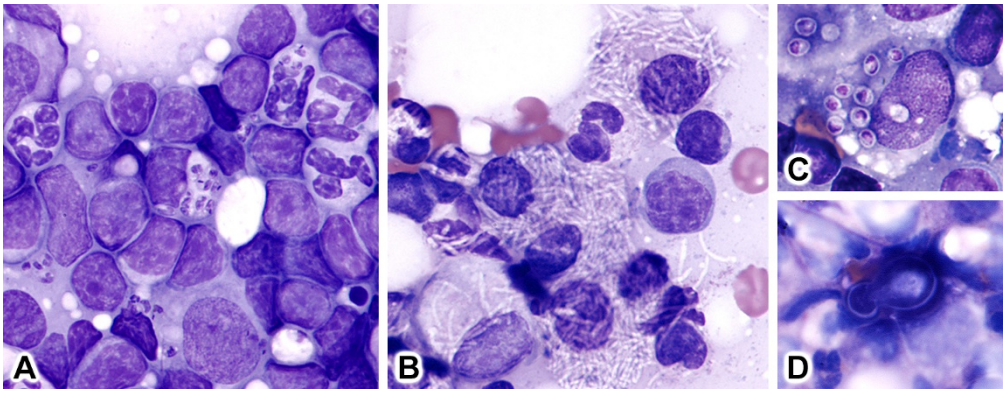


Figure 6.15. A. *Leishmania* sp. amastigotes with rod-shaped kinetoplasts in macrophages and neutrophils, protozoal lymphadenitis, dog. B. Macrophages and neutrophils laden with nonstaining bacilli of *Mycobacterium* sp., mycobacterial lymphadenitis, cat. C. Yeast of *Histoplasma capsulatum* free and in a macrophage, fungal lymphadenitis; despite the species name, the organism lacks a capsule but has a prominent nonstaining cell wall typical of yeast. D. Budding yeast of *Blastomyces dermatitidis* overlying cells in a different focal plane, fungal lymphadenitis, dog.

E. Idiopathic sterile glucocorticoid-responsive lymphadenitis has been reported in dogs, particularly English springer spaniels.[203,204] Inflammation may be neutrophilic, macrophagic, or mixed, with or without necrosis. Pyrexia and lethargy are common, and response to therapy is rapid.
F. Lymphadenitis is often associated with reactive changes, and the combination term *reactive lymphadenitis* is sometimes used to reflect both changes.

IV. Nodal lymphoid neoplasms (lymphoma and plasma cell neoplasms)
A. Anatomic classification, histologic classification, and staging of lymphoid neoplasms are beyond the scope of this book.
B. Lymphoma (also called lymphosarcoma, especially historically and in ruminants) encompasses a group of heterogeneous disorders that develop from different cellular alterations in different subtypes of lymphocytes in different organs, thus yielding diverse clinical presentations with a wide array of cytologic and histologic appearances that vary with the species.
C. Lymph nodes are commonly affected, and lymphoma is commonly diagnosed by cytologic evaluation of lymph node samples, particularly in dogs. Even within the group of canine lymphomas affecting lymph nodes, there are many variations in cytologic features that can relate to the following.
1. General cell type: precursor B lymphocytes, precursor T lymphocyte, mature B lymphocyte, mature T lymphocyte, NK cell
2. Lymphocyte origin/subtype: intermediate-size marginal zone lymphocytes; intermediate-size mantle zone lymphocytes; large centroblasts, large immunoblasts, and smaller centrocytes from germinal centers
3. Distribution pattern: diffuse versus follicular or nodular
D. Cytologically, lymphoma generally can be diagnosed when there is nearly a single (monotypic) population of atypical lymphocytes rather than the heterogeneous mixture of

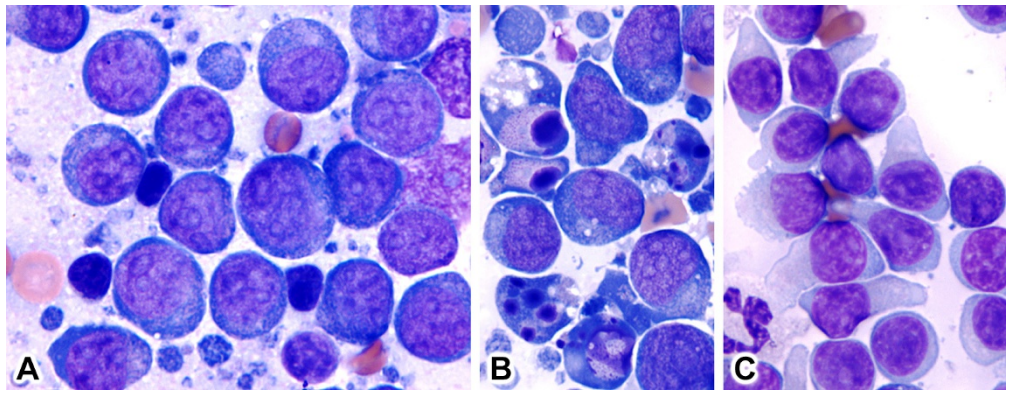


Figure 6.16. A. Predominantly intermediate and large lymphocytes (compare to well spread small lymphocyte at bottom and RBC at left), lymphoma, dog. B. Pyknotic cell death in many neoplastic lymphocytes one day after glucocorticoid therapy for lymphoma, dog. C. Small to intermediate neoplastic lymphocytes, lymphoma, dog.

typical cell types present in health (small lymphocytes predominating) and reactive or inflamed lymph nodes. However, depending on the appearance of the cells, lymphoma can be an easy or difficult diagnosis cytologically.

1. When good-quality cytologic preparations consist of single populations of large lymphocytes with blue cytoplasm and prominent nucleoli, the diagnosis of lymphoma is usually clear (Figure 6.16A), although bovine reactive lymph nodes can mimic lymphoma. This pattern is common because diffuse large B-cell lymphoma is the most common form of lymphoma in dogs.
2. The diagnosis is more difficult (requires an expert and excellent preparations, and still there is substantial interobserver variation) when the cells are small to intermediate in size or when substantial numbers of nonneoplastic cells are mixed with neoplastic cells because of a nondiffuse form or a recent onset (Figure 6.16B). In these cases, histologic examination may be necessary, if not also immunophenotyping or PARR.
3. A lymph node is unlikely to be reactive and likely to be lymphomatous if > 50 % of the lymphoid cells are large lymphocytes, particularly if the populations are largely dichotomous (mostly a population of large cells mixed with typical small lymphocytes) without the heterogeneity expected in a reactive lymph node (additional intermediate and reactive lymphocytes and plasma cells).

E. Over the past 50+ years, the following classification systems, provided in chronologic order, have been developed for human lymphomas. Although used for veterinary species, their applicability to nonhuman lymphomas has been inconsistent. Ideally, a classification system would differentiate neoplasms of differing clinical behavior and prognosis such that they could receive the most appropriate management.

1. Rappaport classification: based primarily on sizes of the lymphocytes and patterns of cell growth (nodular and diffuse)
2. Kiel classification: based on lymphocyte sizes and nuclear and cytoplasmic features; separates lymphomas into B-lymphocyte and T-lymphocyte neoplasms
3. Lukes–Collins classification: based on sizes and shapes of nuclei (e.g., small, large, cleaved, noncleaved, and convoluted) and on cellular features (small, immunoblastic, plasmacytoid, and histiocytic); separates lymphomas into B-lymphocyte and T-lymphocyte neoplasms

4. The Working Formulation system: considers clinical outcome and uses growth patterns (follicular and diffuse), nuclear outline (cleaved, noncleaved, and convoluted), and cell sizes to classify the lymphomas into three grades (low, intermediate, and high)
5. Revised European/American Lymphoma (REAL) system: divides lymphomas into B-lymphocyte or T-lymphocyte disorders on the basis of immunophenotyping, cell structure, tissue architecture, genetic features, and clinical features
6. WHO classification: modified REAL system incorporating aspects of the prior classifications (B lymphocyte, T lymphocyte, and NK lymphocyte);[205] a similar veterinary histologic classification of lymphoid neoplasms in domestic animals was published by the AFIP in cooperation with the American Registry of Pathology and the WHO Collaborating Center for Worldwide Reference on Comparative Oncology[130,131]

F. Canine and human lymphomas have been compared[132] and the WHO classification system has been applied to common types of canine lymphoma with reasonable success.[131] Refinements are likely. It should be noted that cell appearances, including chromatin patterns, vary substantially between histologic and cytologic preparations, so care must be taken to relate published descriptions to the applicable diagnostic approach.

G. Lymphomas can be evaluated by using methods described previously (see Bone Marrow Classifications, Section IX.E) for other hemic neoplasms, the discussed cytochemical techniques being useful to help identify nonlymphoid lineages, thereby excluding lymphoma. Features relevant to cytologic recognition of the major subtypes of canine nodal lymphoma are provided in Table 6.8. Treatment may be instituted based on a cytologic diagnosis with or without immunophenotyping by flow cytometry or immunocytochemistry, but definitive and complete classification generally requires histopathology ± immunohistochemistry.
   1. Microscopic evaluation of stained cells is the easiest method of evaluation and has the most clinical application, but classification of lymphoma is limited without further information.[206] Microscopic appearance (Table 6.8) does not consistently differentiate B lymphocytes and T lymphocytes or predict genetic abnormalities, but it does provide information important for classification.
   2. Immunophenotyping is used primarily to differentiate B-lymphocyte lymphomas from T-lymphocyte lymphomas, but it may also help differentiate nongranular acute lymphoblastic leukemias (usually CD34+) from lymphoma with bone marrow and blood involvement. However, a small percentage of canine B-lymphocyte lymphomas are CD34+.[138,207]
      a. At a minimum, when lymphocytes are immunophenotyped, a B-lymphocyte marker and a T-lymphocyte marker should be assessed (often CD79a and/or CD21 for B lymphocytes and CD3 or CD3ε for T lymphocytes). T-lymphocyte lymphomas have a poorer prognosis,[207] although initial therapy may be the same.
      b. Direct cytologic smears may be used for immunocytochemistry. Some laboratories may also accept previously stained smears, but labeling intensity may be mildly to less often substantially reduced.[208] Alternatively, cells can be aspirated and expressed into an appropriate buffer for evaluation by a number of techniques: (i) immunocytochemistry using concentrated preparations of cell suspensions, (ii) immunohistochemistry on cell blocks made from cell suspensions by pelleting and fixing cells,[209] or (iii) flow cytometry.[210] Immunohistochemistry may also be used on frozen or fixed tissue sections.

Table 6.8. Features of major types of canine nodal lymphoma

Type of lymphoma	Growth pattern[a]	Size[b]	Typical major cytologic features (Wright stains)	Mitotic rate
B Lymphocyte				
Diffuse large B-cell lymphoma*	D	L (I)	Blue cytoplasm, fine chromatin with:	High (most)
Immunoblastic			- single central nucleoli	
Centroblastic			- multiple peripheral nucleoli	
T-cell–rich B-cell (rare)			- minority of large B lymphocytes	
Anaplastic (rare)			- pleomorphism (variability)	
Marginal zone lymphoma	ND	I (S)	Cytoplasm not scant, somewhat dispersed chromatin, single central nucleolus	Low
Mantle cell lymphoma	ND	S to I	Somewhat clumped chromatin, small or inapparent nucleoli	Low (most)
B-cell lymphoblastic leukemia/lymphoma	D	S to I	Round or convoluted nuclei with fine to clumped chromatin and vague or inapparent nucleoli	Very high
Follicular lymphoma	ND	S and L	Mixed: many smaller centrocytes with scant pale cytoplasm, irregular/indented nuclei, moderately dense but dispersed chromatin, no nucleoli; fewer larger centroblasts (see Centroblastic above)	Low
T Lymphocyte				
Peripheral T-cell lymphoma, not otherwise specified*	D	I to L	Variable: pleomorphic, indistinct to prominent nucleoli	Variable
T-zone lymphoma	ND	S to I	Pale cytoplasm, somewhat clumped chromatin, few nucleoli; hand-mirror cells common	Very low
T-cell lymphoblastic leukemia/lymphoma	D	S to I	Round to mildly convoluted nuclei, fine to clumped chromatin, vague or inapparent nucleoli	Very high

* Relatively common subtype

[a] Typical nodal distribution: diffuse (D) or nondiffuse (ND), ND being nodular or follicular so cytologic samples are often mixed

[b] Reported nuclear sizes are small (S), intermediate (I), or large (L); parenthetic sizes have been less commonly described. Inadequately flattened cells are common and appear smaller with less cytoplasm, denser chromatin, and darker cytoplasm.

Note: Many lymphomas are indistinguishable with routine cytologic evaluation alone. Uncommon subtypes that were omitted but add to the described variation include large granular lymphocyte leukemia/lymphoma, T-cell prolymphocytic/lymphocytic lymphoma, lymphoplasmacytic lymphoma, and B-cell chronic lymphocytic leukemia/prolymphocytic/lymphocytic lymphoma.

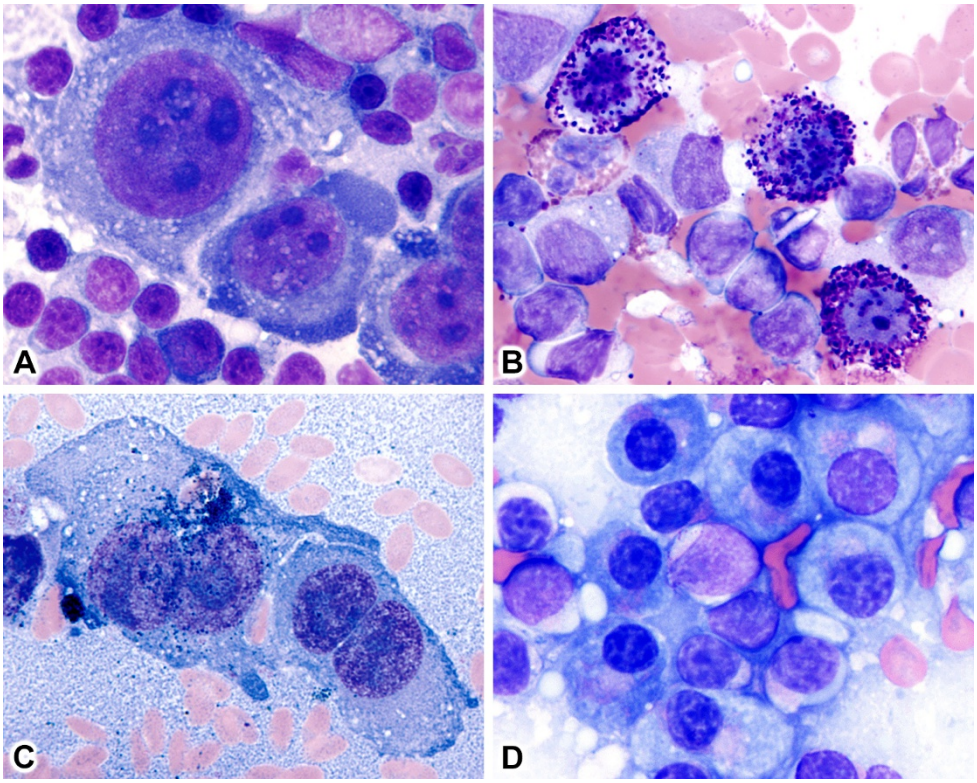


Figure 6.17. A. Small raft of pleomorphic epithelial cells and mostly small lymphocytes, metastatic carcinoma, dog. B. Metastatic mast cells, eosinophils, and lymphocytes, dog. C. Atypical melanoblasts with abnormal melanin granules, metastatic melanoma effacing an axillary lymph node, alpaca. D. Plasma cells mixed with small lymphocytes; proved to be neoplastic, not reactive, and associated with plasma cell myeloma, same dog as in Figure 6.11A.

c. Coexpression of CD3 and CD21 or CD79a has been reported,[207] with the neoplastic cells having had either TCR or immunoglobulin receptor gene rearrangements. This aberrant expression may be a useful diagnostic marker for malignancy.

d. Lymphomas may also be assessed for p53 tumor-suppressor protein, which has been significantly increased in high-grade lymphomas.[211]

3. PARR testing can aid in detection of lymphoma, particularly T-zone, follicular, and T-cell–rich B-cell lymphomas, but it should not be used without microscopic characterization (see Methods of classifying hemic cell neoplasms in Bone Marrow Classifications, Section IX.E.6). It is likely that other cytogenetic and molecular techniques will be of diagnostic and prognostic value.

H. Plasma cell neoplasms may involve lymph nodes (Figure 6.17D), but concurrent sites of involvement are expected. If present, they must be differentiated from reactive lymph nodes when the cells are relatively well differentiated, and from plasmacytoid lymphoma when the cells are less well differentiated (more lymphocytic). Mott cells may be present and even prominent in either reactive or neoplastic processes. *Mott cells* are plasma cells containing multiple cytoplasmic inclusions of immunoglobulin (*Russell bodies*), mostly

within rough endoplasmic reticulum. Russell bodies may also occur as single large inclusions within plasma cells, but such plasma cells do not fit the original description of Mott (morula) cells with multiple inclusions. Similar dilated organelles containing immunoglobulin may invaginate into the nucleus or overlie it to form the pseudoinclusions called *Dutcher bodies*.[212]

V. Nonlymphoid neoplasms (typically metastatic)
   A. Lymph nodes can be enlarged because of the growth of nonlymphoid neoplastic cells in the node, with or without concurrent lymphoid reactivity or inflammation. Metastatic cells can also be found in samples from lymph nodes that do not appear enlarged.
   B. Many neoplasms have the potential to spread to regional lymph nodes. Those seen more frequently in lymph nodes include squamous cell carcinoma, mammary carcinoma or adenocarcinoma, apocrine gland adenocarcinoma of the anal sac, melanoma, mast cell neoplasms, and other hemic neoplasms (Figure 6.17 A–C).

VI. Other findings
   A. Edema may be suggested in aspirates by the loose arrangement of cells in a stained residue that suggests dispersion of cells by fluid. Edema may cause or contribute to lymphadenomegaly.
   B. The presence of nonlymphoid hemic precursors (rubricytes, megakaryocytes, and granulocytes) indicates local hematopoiesis or, rarely, nonlymphoid hemic neoplasms involving the lymph node.
   C. The presence of RBCs in the sample indicates hemorrhage. If only RBCs are found, it may be impossible to differentiate pathologic hemorrhage from hemorrhage caused by sampling. The presence of macrophages containing RBCs and hemosiderin supports the conclusion of pathologic hemorrhage either within the lymph node or its drainage field.
   D. A diagnosis of metastatic melanoma should be considered any time melanin is found. However, melanin can be found in macrophages (melanophages) when the lymph node's drainage field contains a melanoma, necrosis, or inflammation of pigmented tissue.
   E. Various combinations of multiple abnormalities may be present.

References

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Chapter 7

PROTEINS

Kristen R. Friedrichs, Michael A. Scott, and Steven L. Stockham

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Table 7.1. Abbreviations and symbols in this chapter	
$(TP:Fib)_p$	Plasma total protein to fibrinogen
[x]	x concentration (x = analyte)
αAG	α_1-Acid glycoprotein
AGE	Agarose gel electrophoresis
A:G ratio	Albumin-to-globulins ratio
AL	Amyloid light chain
BCG	Bromocresol green
BCP	Bromocresol purple
Cp	Ceruloplasmin
COP	Colloid osmotic pressure
CRP	C-reactive protein
CZE	Capillary zone electrophoresis
ECF	Extracellular fluid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fbg	Fibrinogen
FcRn	Neonatal Fc receptor
Hp	Haptoglobin
Hgb	Hemoglobin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITPI	Inadequate transfer of passive immunity
MGUS	Monoclonal gammopathy of undetermined significance
M_r	Relative molecular mass
NAPP	Negative acute-phase protein
Na_2SO_3	Sodium sulfite
PAPP	Positive acute-phase protein
PCR	Polymerase chain reaction
PLE	Protein-losing enteropathy
PLN	Protein-losing nephropathy
PP:F	Plasma protein to fibrinogen
pTP_{ref}	Plasma total protein by refractometry
RI	Reference interval
RID	Radial immunodiffusion
SAA	Serum amyloid A
SI	Système international d'unités (International System of Units)
SPE	Serum protein electrophoresis
sTP_{ref}	Serum total protein by refractometry
TP_{ref}	Total protein by refractometry
TP	Total protein
WRI	Within reference interval
$ZnSO_4$	Zinc sulfate

GENERAL CONCEPTS FOR TOTAL PROTEIN, ALBUMIN, AND GLOBULINS

I. Physiologic processes (Figure 7.1)
   A. Proteins are polypeptide chains of amino acids joined together by peptide bonds that link the nitrogen-containing amino group of one amino acid to the carboxylic acid group of its neighbor. Over 1000 individual proteins have been characterized in serum. Most are not biochemically pure proteins; they are proteins combined with other substances. For example, lipoproteins are composed of proteins, triglyceride, and cholesterol, whereas glycoproteins contain proteins and polysaccharides (sugar).
   B. Plasma and serum contain albumin and globulins. All proteins other than albumin are globulins, whether immunoglobulins, PAPPs including complement proteins, polypeptide hormones, cytokines, enzymes, proenzymes, or nonenzymatic proteins involved in hemostasis.
   C. A major difference between serum and plasma is that serum, formed from plasma after clotting, does not contain Fbg, which accounts for approximately 5 % of plasma [TP] in health. Serum lacks other clotting factors as well, but they do not contribute significantly to routinely measured protein concentrations.
   D. Most plasma proteins (albumin and most globulins) are synthesized by hepatocytes. The plasma half-life of albumin varies among species and generally increases with species' body size; it is about 5–12 d in dogs[1,2] and about 2–3 wk in cattle and horses.[1,3,4] It is also inversely related to [albumin], being much longer in analbuminemic people than in healthy people. There is a large variation in the half-life of the various proteins in the globulin fraction, and there are very few publications regarding half-lives for the plasma globulins in domestic mammals.
   E. Most globulins not produced by hepatocytes are immunoglobulins (Figure 7.2). Immunoglobulins are produced by B lymphocytes and plasma cells. They are classified by their heavy chains as IgG, IgM, IgA, or IgE. Subclassifications also exist.
      1. Llama and alpaca IgG includes about 50 % IgG molecules containing both light and heavy chains (IgG_1) and 50 % IgG molecules that lack light chains (IgG_2 and IgG_3) but also function to bind antigen.[5,6]
      2. Data for domestic species are scant, but plasma immunoglobulin half-lives appear to vary from days to a few weeks, with those of IgG greater than those of IgM and IgA. The average half-life of colostral IgG in foals was 26 d.[7] This contrasts with a reported mean IgG half-life for colostral IgG in cats of about 4–5 d.[8]
   F. Proteins continuously undergo degradation in vivo, necessitating continuous synthesis and therefore availability of amino acids. The reason for the relatively long half-lives of IgG and albumin compared to many proteins appears to be a shared anti-degradation mechanism rather than inherent properties of the proteins. When IgG and albumin enter cells through pinocytosis, they bind to FcRns within endosomes where the pH is low. FcRn was named for its involvement in the transfer of IgG to neonates. The complexes are then exported from the cell where the more neutral pH of blood or interstitial fluid dissociates IgG and albumin from FcRns, effectively rescuing the molecules from degradation. Many cells express FcRns, including endothelial cells, macrophages, dendritic cells, neutrophils, and epithelial cells.[9,10]
   G. Albumin is the single most abundant plasma protein, accounting for approximately half of the total plasma protein. Even more albumin is present in interstitial fluid, though at much lower concentrations. An albumin molecule recirculates from blood to tissue to lymphatics and back to blood many times before its degradation, providing several functions along the way.[11]

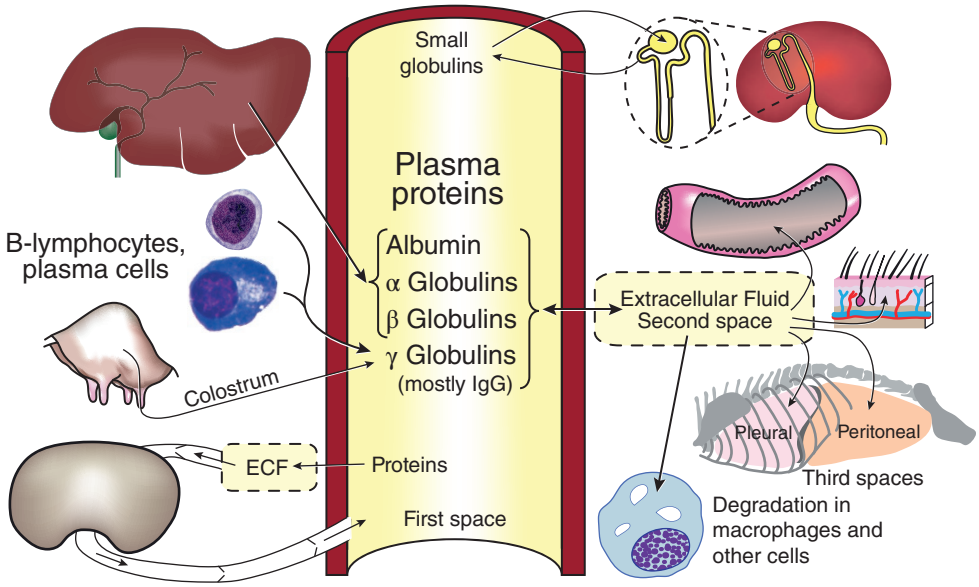


Figure 7.1. Physiologic factors that influence serum or plasma protein concentrations. Plasma proteins are either albumin or globulins; globulins are either immunoglobulins or other globulins. Globulins can be divided by their electrophoretic migration into α, β, and γ fractions.

- Albumin: Hepatocytes synthesize albumin and transport it extracellularly. It then passes through the fenestrated hepatic sinusoids into blood where it is the main contributor to COP. A small percentage of albumin enters the ECF of interstitial tissue throughout the body and serves as the main contributor to interstitial COP. Albumin that is not lost from the body or catabolized recirculates to blood via lymphatics. Albumin may be degraded by many cell types, but degradation appears to be reduced through intracellular rescue and export via FcRn recycling, thus prolonging albumin's half-life.
- Immunoglobulins: Plasma immunoglobulins are derived from the ingestion of colostrum by the neonate before "gut closure" and from plasma cells and B lymphocytes that produce antibodies as part of acquired immunity throughout life. IgG predominates from both sources and migrates electrophoretically as a γ globulin, whereas other immunoglobulin types are β or γ globulins. Immunoglobulins are thought to extravasate to interstitial tissue largely via paracellular pores, some tissues (e.g., liver, spleen, bone marrow) having greater porosity than others. Immunoglobulins not catabolized in tissues enter lymph and are returned to blood. Catabolism may occur through receptor-mediated endocytosis after binding to target epitopes, by phagocytosis of immune complexes, or by endocytosis and catabolism by many cell types. However, FcRn binding in endosomes redirects IgG for release from the cell, thus prolonging the half-life of IgG.
- Other globulins: Most of the mass of plasma globulins other than immunoglobulins is synthesized by hepatocytes, important types of globulins being APPs (including complement proteins) and coagulation factors or other proteins involved in hemostasis, but many globulins (e.g., cytokines, polypeptide hormones, von Willebrand factor, and some enzymes) produced by other cells also circulate in low concentrations. These proteins may be α, β, or γ globulins. Some are smaller than albumin and pass freely through the glomerular filtration barrier; some of these are lost in the urine and some proteins are resorbed by receptor-mediated endocytosis in the proximal tubules. As for albumin and immunoglobulins, some of these globulins enter interstitial tissue to carry out their functions, be degraded, or return to blood via lymphatics.
- Other than hemorrhage anywhere in the body, the major sites of potential protein loss from vessels are the kidneys, intestine, skin, and cavitary spaces (third space loss).

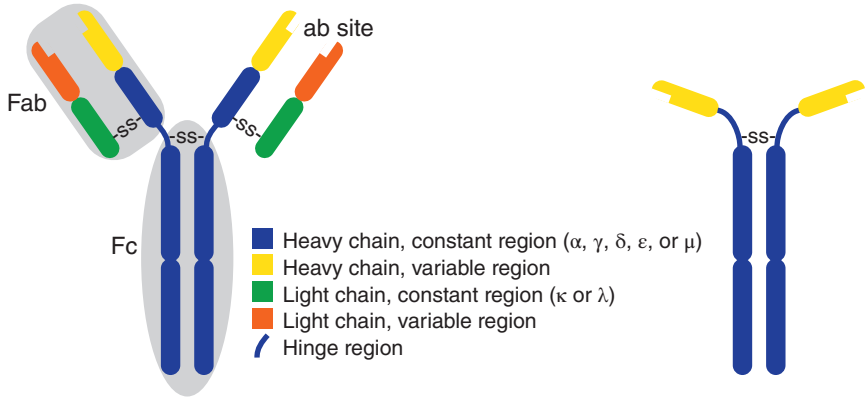


Figure 7.2. Schematic structure of a typical mammalian immunoglobulin molecule (*left*) and IgG_2 or IgG_3 from camelids (*right*).

- A typical immunoglobulin molecule consists of two heavy chains of the same class (α for IgA, γ for IgG, δ for IgD, ε for IgE, and μ for IgM) and two light chains (either κ or λ but not both). The two heavy chains are linked by disulfide bonds in the hinge region. Each light chain is linked to a heavy chain by a disulfide bond. Each combined heavy and light chain variable region forms an antigen binding (ab) site. Enzymatic cleavage on the Fab side of the disulfide bonds in the hinge region generates the crosslinked base of the Y-shaped molecule consisting of vertical segments of two heavy chains; this is called the Fc fragment (Fragment, crystallizable) and is highlighted within a gray area. Cleavage of the slanted portions of the Y-shaped molecule forms Fab fragments (Fragments, antigen binding), also highlighted in a gray area. IgA molecules may be linked by a J chain to form dimers, and IgM molecules are linked to form pentamers (not shown).
- In addition to typical immunoglobulins, camelids also synthesize functional IgG with only heavy chains. The heavy chains are linked together by disulfide bonds in the hinge region. IgG_2 and IgG_3 differ in the length of the hinge region and the composition of the constant regions. Antigen binding occurs via the heavy chain variable region.

1. Although albumin is responsible for about 50 % of the plasma protein concentration, it contributes to a greater percentage of the serum COP (approximately 80 %) because of its relatively small size and high negative charge (Donnan effect) (see Colloid Osmotic Pressure). This helps maintain intravascular fluid volume.
2. Albumin and other proteins contribute to the nonbicarbonate buffering capacity to help maintain blood pH.
3. Albumin provides amino acids to cells throughout the body after uptake and degradation; the amino acids can be used for protein synthesis or energy. Liver, muscle, and kidneys are major sites of albumin catabolism.
4. It is a transporter for molecules and ions, including fatty acids, unconjugated bilirubin, steroids, and divalent cations (as well as many drugs).
5. It has antioxidant properties, partly via an exposed unpaired cysteine sulfhydryl group.

H. Other proteins have a wide variety of specific functions such as involvement in hemostasis, immunity, transport, and regulation of metabolic activity, and all contribute to COP.

I. Age-related changes: [TP] is lowest at or very soon after birth when volume expansion decreases protein concentrations in the absence of colostrum. With adequate absorption of good-quality colostrum, plasma concentrations of immunoglobulins and globulins increase abruptly, then decrease as colostral immunoglobulins are cleared over several weeks (e.g., reaching a nadir in kittens at 4–6 wk),[12] and subsequently increase to adult values with increased immunoglobulin production.

1. In puppies and kittens, [globulins] and [TP] may be below adult reference values at 8 wk;[12–14] increases are expected for 1–2 yr[15] and may occur until old age.[16] Similar trends are expected in other species.
2. The [albumin] in puppies and kittens typically increases progressively to adult values over about 6 mo,[12,13,17] but age-related changes in canine [albumin] have not been consistently reported.[15] The [albumin] of calves has been reported to increase over at least the first 6 wk of life.[18] Similar trends are expected in other species.

J. Breed differences: Retired healthy greyhounds (mean age, 5.7 yr) enrolled in a blood donor program had mildly lower mean concentrations of total protein (averaging 0.4–0.9 g/dL lower) and globulins (averaging 0.3–0.4 g/dL lower) than nongreyhound dogs (mean age, 3.8 yr).[17,19] This was shown to be associated with lower α- and β-globulin concentrations and mildly lower concentrations of IgA and IgM. Decreased serum [Hp] in greyhounds compared to other dogs contributes to the lower [α globulins].[20]

II. Protein disorders

A. *Protein dyscrasia* is the presence of an abnormal protein (abnormal structure).

B. *Dysproteinemia* is the presence of a protein dyscrasia or normal protein at abnormal concentration in the blood.

1. Selective or nonselective dysproteinemias
   a. *Nonselective hyperproteinemia*: All protein concentrations are increased (*panhyperproteinemia*). It results from hemoconcentration.
   b. *Selective hyperproteinemia*: The [TP] is increased, and some protein concentrations are increased more than others. Typically, it results from inflammation or a lymphoid neoplasm producing whole or partial immunoglobulin molecules.
   c. *Nonselective hypoproteinemia*: The [TP] is decreased, and all protein concentrations are decreased (*panhypoproteinemia*). Its cause is a proportional loss of all proteins, although coincidental selective processes could yield a proportional decrease in synthesis of albumin and globulins that would appear similar.
   d. *Selective hypoproteinemia*: The [TP] is decreased, and some protein concentrations are decreased more than others. Its cause is selective loss (typically smaller proteins selectively lost) or selectively decreased synthesis of one or more proteins.
   e. Mixed dysproteinemia is common, occurring with concurrent disorders that selectively or nonselectively affect plasma protein concentrations. Some combinations of disorders have additive effects, e.g., inflammation and dehydration both promoting hyperproteinemia. Some concurrent disorders have opposing effects that may even mask a dysproteinemia, e.g., dehydration promoting hyperproteinemia while external blood loss promotes hypoproteinemia.
2. To determine whether the dysproteinemia is selective or nonselective, SPE may be needed to evaluate the relative concentrations of protein groups, especially in the globulin regions. An animal with hypoproteinemia, hypoalbuminemia, and hypoglobulinemia may or may not have nonselective hypoproteinemia.
   a. If hypoalbuminemia and hypoglobulinemia are present and electrophoresis results indicate that all protein fractions are decreased proportionately, then there is a nonselective hypoproteinemia.
   b. If hypoalbuminemia and hypoglobulinemia are present and electrophoresis results indicate that protein fractions are decreased disproportionately, then there is a selective hypoproteinemia.
   c. The same concepts apply to hyperproteinemia evaluations.

C. Selective dysproteinemia commonly results from alterations in specific protein concentrations during inflammatory diseases. There are three major groups of proteins whose plasma concentrations change because of inflammation.

1. *PAPPs*, a subset of positive acute-phase reactants that include molecules other than proteins, are those proteins that have increased local, plasma, or serum concentrations because of increased production in response to inflammatory cytokines (e.g., IL-6). Hepatocyte synthesis is primarily responsible for increased blood concentrations of these acute-phase reactants, although extrahepatic production can cause increases for some, particularly locally (e.g., in synovial fluid or milk).
   a. PAPPs are often categorized as major, moderate, or minor based on their magnitude of increase with inflammation (Table 7.2).[21–23]
      (1) Concentrations of *major PAPPs* are low in healthy animals, rise dramatically to as much as 10 to 100 times baseline upon stimulation, peak in 24–48 h, and decline rapidly during recovery.
      (2) Concentrations of *moderate PAPPs* increase to as much as 10 times baseline upon stimulation, peak after 2–3 d, and decline more gradually.
      (3) *Minor PAPP* concentrations gradually increase up to twice the resting level.
   b. Commonly measured PAPPs and their major functions
      (1) *Fibrinogen* (Fbg, $M_r \cong 340{,}000$), the precursor of fibrin, is used to form secondary hemostatic plugs at sites of vascular injury. Fbg, fibrin, and their fragments also have important roles in wound healing and the promotion and resolution of inflammation. Fbg is not in serum.
      (2) *C-reactive protein* (CRP, $M_r \cong 115{,}000$), named for its ability to bind to capsular polysaccharide (C-polysaccharide) of *Streptococcus pneumoniae*, promotes binding of complement to bacteria with subsequent opsonization and destruction.
      (3) *Serum amyloid A* (SSA, $M_r \cong 14{,}000$) proteins are hydrophobic apolipoproteins associated with high-density lipoproteins; they induce synthesis of several cytokines and promote recruitment of inflammatory cells (neutrophils, mast cells) to inflammatory sites.

Table 7.2. Positive acute-phase proteins (PAPPs) in common domestic mammals

Species	Major[a]	Moderate[a]	Minor[a]
Dogs	SAA, CRP	Hp, αGP, Fbg	Cp
Cats	SAA, αAG	Hp, Fbg	Cp
Horses	SAA	CRP, Fbg, Hp	αAG, Cp
Cattle	SAA, Hp	αAG, Fbg, Cp	CRP[b]
Alpacas/llamas[c]	?	Fbg, Hp	?
Pigs	SAA, Pig-MAPP, Hp, CRP	Fbg	?

[a] PAPPs are classified based on the greatest documented magnitude of increase compared to baseline values or values in healthy animals of the specified species. Major, > 10×; Moderate, > 2× but < 10×; Minor, < 2×. Most responses for a PAPP may fall in a lower classification level. Some moderately responsive PAPPs may be commonly used in a species (e.g., CRP in horses).
[b] Some do not consider CRP a PAPP in cattle, but acute-phase increases have been reported.
[c] SAA was not detected in one study.[24] No data were found for Cp, CRP, or αAG.
Pig-MAPP, porcine major acute-phase protein

(4) *Haptoglobin* (Hp, $M_r \cong 100{,}000$ but forms polymers) binds free Hgb dimers from hemolysis so that iron is not available to organisms and is recycled rather than excreted. Removal of Hgb from circulation also limits oxidative damage from the peroxidase activity of Hgb.

(5) *α_1-Acid glycoprotein* (αAG, $M_r \cong 43{,}000$), also called orosomucoid, is a highly glycosylated protein known to transport neutral and basic lipophilic compounds in the blood. It also has several anti-inflammatory activities.

(6) *Ceruloplasmin* (Cp, $M_r \cong 115{,}000$–$135{,}000$) is an enzyme that transports copper, has oxidase activity, and affects iron homeostasis. It helps convert ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}) for transport in the plasma as transferrin.

(7) *Ferritin* ($M_r \cong 450{,}000$) is important for iron homeostasis. It serves as a storage form of iron (in Fe^{3+} form). Most ferritin is in tissues, but small amounts leave cells and enter plasma.

c. Other PAPPs include complement proteins, hepcidin, procalcitonin, serum amyloid P, mannan-binding lectin, α_2-macroglobulin ($M_r \cong 820{,}000$), α_1-antitrypsin, α_1-antichymotrypsin, and thymidine kinase (see Chapter 12 for thymidine kinase).

2. *NAPPs* are those proteins that have decreased plasma or serum concentrations because of an inflammatory process. Their concentrations decrease because of decreased production by hepatocytes due to the actions of cytokines (e.g., IL-6 and IL-1).
   a. Albumin ($M_r \cong 69{,}000$) is the only commonly evaluated NAPP.
   b. Transferrin, the combination of apotransferrin and iron, is typically measured as total iron binding capacity in the assessment of iron status, not inflammation (see Chapter 3); total iron binding capacity (transferrin) may be decreased because of ongoing inflammation.[25]
   c. Other NAPPs include paraoxonase-1, adiponectin, IGF-1, and transthyretin; there may be species differences in response to inflammation.
3. *Immunoglobulins* are *delayed-response proteins* that may noticeably increase serum or plasma [TP] and [globulins] by 1–3 wk after the onset of inflammation. Increased immunoglobulin concentrations may persist for months.

ANALYTICAL PRINCIPLES FOR TOTAL PROTEIN, ALBUMIN, AND GLOBULINS

I. [TP]
   A. Refractometry for measuring [TP] (plasma or serum): Determination of the [TP_{ref}] is part of a complete blood count (CBC) in some veterinary laboratory settings, allowing for the concurrent visual inspection of plasma character in a microhematocrit tube.
      1. Principle: The degree of light refraction in an aqueous solution is proportional to the concentration of solids in solution, and this refraction can be measured with a refractometer. Because most solids in plasma are proteins, the degree of light refraction is highly dependent on protein concentration, and a refractometer's total protein scale is calibrated with the assumption that changes in refractive index reflect changes in protein concentration alone. However, other solutes can affect sample refraction.
      2. Because light refraction is a physical property, the [TP] determined via refractometry may not be the same as determined by a biuret reaction (clinical chemistry method). In fact, it is frequently mildly different (≤ 0.3 g/dL) and not uncommonly higher by as much as 2.0 g/dL in samples that are not hemolyzed, icteric, or lipemic.
      3. Refractometers

a. A temperature-compensated refractometer is recommended over a non–temperature-compensated refractometer for two reasons:
   (1) It does not require daily adjustments based on ambient temperatures.
   (2) It will probably be more accurate. If compensated and noncompensated refractometers are calibrated to agree at 68 °F, they will disagree by about 0.3 g/dL at 75 °F and by about 0.7 g/dL at 85 °F (Leica TS400 Total Solids Refractometer literature).
b. With traditional analog refractometers, external light passing through the sample layer on a prism is bent and focused on a calibrated internal scale reticle that is assessed visually through a magnifying eyepiece. The [TP_{ref}] is the level on the scale that is crossed by the light/dark interface (shadow line). Temperature compensation occurs via a bimetal strip that moves and adjusts lenses in response to temperature changes.
c. With digital refractometers for [TP_{ref}], light from an internal source is focused on the surface of a prism where sample is applied, and the pattern of reflected light is detected by internal sensors that convert the signals to a digital value. Temperature correction factors can be automatically applied based on an internal thermistor (temperature-dependent resistor).
d. Most refractometers are calibrated for the proteins in plasma from healthy people. The calibration scale varies among species because of the different composition of plasma proteins,[26] but the difference is typically considered clinically insignificant.

4. Unit conversion: g/dL × 10 = g/L (SI unit)[27]
5. Interferences: Interferents that falsely increase [TP_{ref}] commonly mask hypoproteinemia or suggest hyperproteinemia when it is not present.
   a. Because the refractive index of a solution depends on the percentage of total solids (by weight) in the sample, markedly increased concentrations of a variety of substances (e.g., glucose, urea, Na^+, and Cl^-, and lipids) could increase the refractive index and thus the total protein reading.
      (1) The total protein reading is reported to be falsely increased by 0.6 g/dL if the plasma [glucose] is approximately 700 mg/dL (0.7 g/dL) or the [UN] is approximately 300 mg/dL ([urea] approximately 0.64 g/dL).[28,29] Increases in the [TP_{ref}] caused by increases in measured solutes can be estimated by first converting a solute's concentration to g/dL (for UN results, this requires converting [UN] to [urea]) and then converting the g/dL to TS % (g/100 g) by dividing the solute concentration by 0.938 (approximate percentage of serum water when [TP] is 7.0 g/dL).
      (2) Gross lipemia increases the refractive index and thus falsely increases the total protein reading; the effect of lipids is not predictable because of the various types of lipoproteins. Increased [cholesterol], reflecting increased [lipoprotein], is associated with false increases in [TP_{ref}] in the absence of gross lipemia.[30,31]
   b. Hemolysis causing a plasma [Hgb] of 0.5 g/dL (500 mg/dL, marked hemolysis) did not interfere with refractive index values but made reading of the shadow line in the refractometer more difficult.[26]
   c. Although icterus is commonly listed as a cause of falsely increased [TP_{ref}] in clinical chemistry textbooks, addition of unconjugated bilirubin to serum at final concentrations of up to 41.5 mg/dL did not affect [TP_{ref}], and similar concentrations of conjugated bilirubin or any other solute would have negligible impact on [TP_{ref}].[28] Clinically suspected effects of bilirubin on [TP_{ref}] may relate to

documented bilirubin-mediated false decreases in chemical (biuret) protein concentrations or to concurrent increases in other unrecognized substances that affect sample refraction.[32]

d. Synthetic colloids such as hydroxyethyl starch and dextran contribute a small amount to refractive index, and thus to [TP_{ref}], as documented by adding them in therapeutic concentrations to simulated hypoalbuminemic solutions.[33] In mixing studies using succinylated gelatin, a protein colloid, the solution contributed substantially to [TP_{ref}] when it was at least 20 % of the sample (and perhaps less);[34] although such [TP_{ref}] values reflect [TP], they do not assess a patient's own concentrations of albumin and globulins. False increases in [TP_{ref}] occurred for up to about 3 h in greyhounds after administration of 20 mL/kg of 6 % hydroxyethyl starch 130/0.4 over 20 min for experimental hemorrhagic shock, peaking within the first hour at about 1.3–2.0 g/dL above chemically determined TP concentrations.[35]

6. Low plasma [TP_{ref}] results are reliably low, except in rare cases of cryoglobulinemia. A rare mismatch between [TP_{ref}] and the serum [TP] determined chemically occurs when cryoprotein is measured in plasma but is absent from serum that was removed after whole blood clotted and cooled. *Cryoglobulins* are proteins that precipitate out of solution at various temperatures below body temperature and resolubilize upon warming. Cryoglobulins may be polyclonal but are more often monoclonal immunoglobulins associated with lymphoproliferative neoplasms.[36]
7. Total protein versus total solids concentration
   a. The [TP_{ref}] is commonly referred to as the *plasma total solids concentration* because the value is affected by solutes other than protein. However, this is not appropriate because most refractometer scales are calibrated for protein concentration rather than total solids concentration (as some are), and thus other substances (e.g., urea or glucose) are interferents in protein assessment rather than the targets of measurement.
   b. The unit for [TS_{ref}] is % or g/100 g (g of solids per 100 g of solution), whereas the unit for [TP_{ref}] is g/dL (g of protein per 100 mL of solution); the units for [TS_{ref}] and [TP_{ref}] are not the same.
      (1) The confusion about units is partially due to the names of some clinical refractometers, e.g., a refractometer may be called a TS meter but have scales calibrated for [TP] rather than [TS].
      (2) Serum with a [TP_{ref}] of 7.0 g/dL has a [TS_{ref}] near 8.4 g/100 g if there are not increased concentrations of electrolytes, urea, glucose, or lipids.[37]
8. Serum [TP_{ref}]: The [TP] in serum (rather than plasma) may also be estimated with a refractometer. The serum [TP] is lower (mildly in health) than the plasma [TP] because of the absence of Fbg in serum. Because solutes of an anticoagulant solution (including anticoagulant and additives) add to the refractive index, plasma [TP_{ref}] may be further increased compared to serum [TP_{ref}] if the collection tube is markedly underfilled, particularly at lower protein concentrations.[38]

B. Biuret reaction for measuring [TP] (serum or plasma)
1. Principle: Copper binding to peptide bonds creates a violet complex; the number of peptide bonds, and therefore amount of color change, is proportional to [TP]. However, not all individual proteins react in the same way, and not all proteins are pure polypeptides that contain the same amount of nitrogen by weight.
2. [TP] in human serum was shown to be stable at 18 °C for 48 h, at 4 °C for 14 d, and at –20 °C for 4 mo in properly handled samples.[39] Evaporation or sublimation of inadequately sealed liquid or frozen samples, respectively, may falsely increase [TP].

3. Unit conversion: g/dL × 10 = g/L (SI unit)[27]
4. Interferences: In some assays, hemolysis may cause a positive interference (e.g., Hgb at 400 mg/dL produces a 12 % bias). Dextran (a polysaccharide used as a plasma expander) may or may not interfere.[40] Succinylated gelatin, a negatively charged (anionic) protein (mean M_r = 26,500) derived from collagen,[41] is also used as a plasma expander; it actually increases the plasma protein concentration, the degree of increase depending on the amount administered, disease state, and timing of sampling after administration.[34] Small plasma peptides may react in the biuret assay but contribute very little to total color change. Ammonium may interfere with the biuret reaction at high concentrations but not at concentrations found in plasma or serum.[42]
5. Increasing degrees of marked hyperbilirubinemia (starting at approximately 10 mg/dL of bilirubin) cause increasing negative interference of TP measurement when using a bichromatic biuret method.[32] In other biuret assays, bilirubin reportedly has no significant effect (package inserts of Roche, Beckman Coulter, and Vitros assays).
6. Comment: The biuret reaction is the most common spectrophotometric method of measuring serum or plasma [TP], but it is not accurate for measuring the much lower protein concentrations in cerebrospinal fluid (see Chapter 21) or urine (Chapter 8).

II. Albumin concentration

A. BCG (G for green) dye-binding reaction (serum or plasma)

1. BCG dye binding is the most common spectrophotometric method of measuring serum or plasma [albumin].
2. Principle: BCG preferentially binds to albumin and produces a color complex. The quantity of BCG–albumin complex is proportional to the [albumin], though binding varies among species; for example, the binding of BCG to bovine albumin is much stronger than to canine and feline albumin.[43]
3. Albumin is stable at 18 °C for 7 d, at 4 °C for 1 mo, and indefinitely at –20 °C if appropriately handled.[39] Evaporation or sublimation of inadequately sealed liquid or frozen samples, respectively, may falsely increase [albumin].
4. Unit conversion: g/dL × 10 = g/L (SI unit)[27]
5. Interferences
   a. Globulins: The binding of BCG to some globulins results in a falsely elevated [albumin]. This nonalbumin binding may lead to significant errors when the true serum or plasma [albumin] is very low (< 1 g/dL) compared to the concentration of interfering globulins (e.g., α_2-macroglobulin).
   b. Hgb: In some assays, Hgb at 0.4 g/dL (400 mg/dL) caused a positive 24 % proportional bias, whereas triglycerides at 0.8 g/dL (800 mg/dL) yielded a constant negative bias (interference) of about 0.2 g/dL.
   c. Anticoagulants: Some BCG methods, but not others, are affected by the presence of anticoagulants. Heparinized plasma yielded a different and most often greater [albumin] compared to serum with a standard BCG assay (median difference, 0.2 g/dL; –0.6 g/dL to 1.2 g/dL), but mostly lower values with a modified assay (median difference, –0.1 g/dL; –0.9 g/dL to 0.1 g/dL).[44] About 50 % of the difference in the standard assay was due to the presence of Fbg in plasma.

B. BCP (P for purple) dye-binding reaction (serum or plasma): BCP binding is used in some human medical laboratories, but albumin molecules of different mammalian species have different affinities for BCP, so BCP assays may give falsely low results in some domestic species (e.g., dogs) while performing adequately in others (e.g., horses).[45] Markedly low

results may be generated if calibration standards from the same species are not used (e.g., human standards for canine samples).[46]

C. HABA (2-[4′-hydroxyazobenzene]-benzoic acid) dye-binding reactions (serum or plasma) are unreliable in domestic mammals[47] but the reagents are still available.

D. Protein electrophoresis can be used to determine [albumin], but it is more often used for quantitating globulin fractions.

III. [Globulins] (methods for immunoglobulins and PAPPs are in later sections)

A. This is typically determined by subtraction.

1. Principle: All proteins in serum other than albumin are globulins.
2. [Globulins] = [TP] – [albumin]

B. Protein electrophoresis can be used to measure [globulins] (see the next section).

C. Unit conversion: g/dL × 10 = g/L (SI unit)[27]

D. Comments

1. [Globulins] is only as accurate as the measured [TP] and [albumin].
2. [Globulins] represents the total concentration of all serum proteins other than albumin (> 1000 proteins, e.g., Hp, transferrin, α_2-macroglobulin, lipoproteins, and immunoglobulins).

IV. Serum protein electrophoresis (SPE) for determining protein fractions

A. SPE is typically requested in a small percentage of cases, but it may be helpful in (i) differentiating causes of hyperproteinemia and hyperglobulinemia, (ii) characterizing hypoproteinemia into selective or nonselective categories, (iii) screening for a monoclonal gammopathy in normoproteinemic and hyperproteinemic sera, and (iv) providing a more accurate estimation of [albumin] when globulins interfere with the BCG assay. SPE results also aid understanding of routine serum concentrations of total protein, albumin, and globulins.

B. As the name indicates, serum is the preferred sample for electrophoresis because plasma [Fbg] is relatively high, and Fbg interferes with detection of protein abnormalities, particularly monoclonal gammopathies. Ethanol precipitation can be used to remove Fbg from plasma samples for the detection of monoclonal bands that may otherwise be masked; however, other proteins may be removed as well, thus affecting quantitation of proteins in each fraction.[48] If electrophoretogram abnormalities remain after removing Fbg, electrophoresis should be repeated with serum.[49]

C. Principle of agarose gel and cellulose acetate electrophoresis

1. Agarose gels have largely replaced cellulose acetate, but either technique separates serum proteins into 4–6 major groups of one or more bands (5–9 with cellulose acetate, 10–15 with agarose) based on their ability to migrate through cellulose acetate or agarose in an electrical field. The degree of migration toward the anode (positively charged terminal) is based on electric charge and a protein's mass and shape. The pH of the electrophoresis medium affects the charge and migration of proteins; the pH is typically basic (8.0–9.0) so that proteins are negatively charged. In domestic mammalian sera, albumin migrates the farthest because it is small and very anionic, though migration rates vary with the species because of differences in amino acid composition and therefore overall charge. Smaller proteins may not migrate as far if they are less negatively charged. Other globulins (e.g., α_2-macroglobulin) are very large but negatively charged enough to cause anodal migration. Electroosmotic forces caused by negative and positive ions in the buffering solution moving respectively toward the

anode and cathode contribute to separation of proteins; these forces limit movement of certain γ globulins toward the anode and force others in a cathodal direction.

2. After electrophoresis, a cellulose acetate strip or agarose gel is stained and scanned with a densitometer. Stained proteins cause less light to be transmitted through the strip to the detector, and decreased transmittance is recorded as a deflection on a densitometer tracing. Scanning yields a curve that reflects the protein content of each fraction (Figure 7.2). The area under the curve represents the total quantity of stained protein. Densitometers may be calibrated so that complete transmittance does not deflect the needle (zero response) and so that the most blockage of light caused by the darkest protein band deflects the needle nearly 100 % (maximum response). The darkest band (or the maximal response) is normally the albumin band but can be found in the globulin fractions. Flatbed scanners obtain digital data that are transformed into relative percentages (e.g., Helena QuickScan 2000).

D. Principle of capillary zone electrophoresis (CZE)
   1. Proteins are separated in a liquid buffer within a narrow capillary tube subjected to high voltage, molecular mobility being dependent on charge and charge to mass ratio.[50–52] Increased automation, narrow bore capillary, liquid medium, and high voltage allow fast and efficient separation of proteins using very small sample volumes. More-positively charged proteins flow more quickly toward the cathode because of charge and electroosmotic forces while more-negatively charged proteins move more slowly because of their charge attraction to the anode. As the protein fractions pass by an ultraviolet light detector (spectrophotometer), protein absorbance is measured and quantified in real time without the need for staining and densitometry.
   2. The digital CZE data are converted to profiles (curves) similar to those of agarose and cellulose acetate but with narrower peaks and humps that are more numerous and spaced differently.

E. Calculating concentrations of the protein fractions: The electrophoretogram is divided into regions (e.g., albumin, α_1, α_2, β_1, β_2, or γ), either automatically or by visual inspection (Figure 7.3). The analyzer calculates the percentage of the total area under the curve for each region to determine the percentage of [TP] represented by each. The protein concentration of each electrophoretic region is then calculated as the product of the [TP] (preferably from a biuret reaction) and the percentage of total protein in each region. For example, if the [TP] is 6.0 g/dL and electrophoresis results indicate that the albumin represents 50 % of the total protein, then the [albumin] is calculated to be 3.0 g/dL (50 % of 6.0 g/dL).

F. Proteins that are the major contributors to the electrophoretic pattern are shown in Figure 7.3, which is based on limited canine data for routine and high resolution AGE.[53,54] Based on limited data, protein migrations are similar among species,[55] but there are species, individual,[56,57] and method variations.

G. Analytical variation and limitations
   1. With any of these electrophoretic methods, the concentrations of protein fractions may be affected by inter-individual differences in defining fraction limits and even in naming them (e.g., β_1 vs β_2 or β_2 vs γ). Also, any inaccuracy in the [TP] affects the accuracy of calculated fraction concentrations.
   2. Agarose and cellulose acetate methods
      a. Different stains have different affinities for some proteins, leading to variation in staining and protein detection. Coomassie brilliant blue and amido black stains have greater capabilities of detecting small amounts of protein than does Ponceau S stain and thus tend to be used in high-resolution electrophoretic systems.

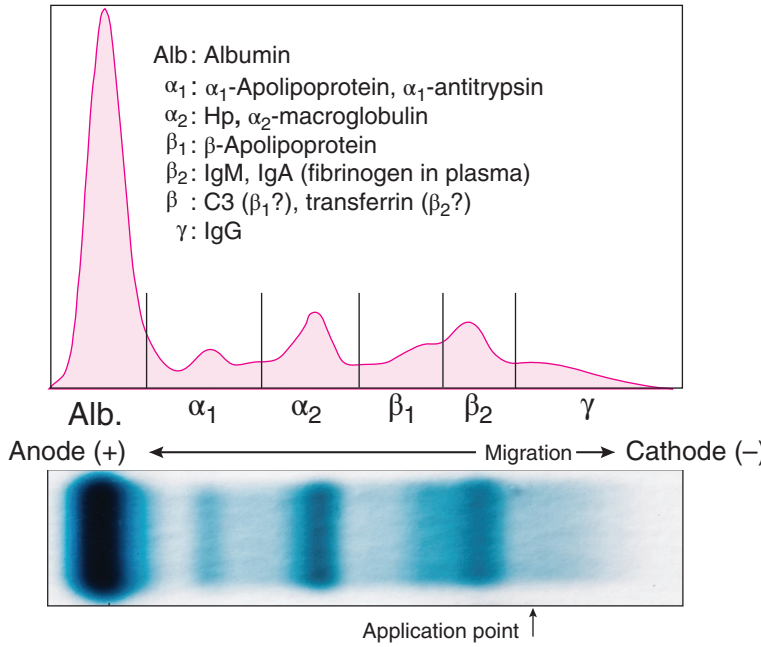


Figure 7.3. Agarose gel SPE with the associated densitometer tracing for a healthy dog.

- Agarose gel (*bottom*): Serum is pipetted into an application well of a gel within an alkaline medium that makes proteins more anionic. The gel is subjected to an electric current to separate proteins based on their electric charge, mass, and shape as they migrate through the gel. Albumin migrates the farthest toward the anode, and globulin fractions separate into bands or fractions: α_1 globulins, α_2 globulins, β_1 globulins, β_2 globulins, and γ globulins. After electrophoresis, the strip is stained with a protein stain (e.g., amido black, as shown). Bands that contain the most protein stain the darkest.
- Densitometer tracing (*top*): When scanned with a densitometer, the tracer pen draws a line that corresponds with the intensity of protein staining. The darkest band causes the highest peak on the tracing, and other peaks or humps are lower depending on the relative staining intensities of the corresponding bands. The relative quantity of protein in each fraction is represented by the area under the curve for each fraction. One peak may represent the staining of essentially one protein (e.g., albumin) or may represent the sum of multiple major proteins (e.g., the α_2 region includes Hp and α_2-macroglobulin). Peaks wider than albumin usually reflect contributions from multiple proteins, but a large amount of one protein could yield a dense band and associated peak that are wider than those for albumin (particularly if hypoalbuminemia is present) simply because of the large amount of protein migrating to the same place.
- Protein fractions and proteins: The band/peak nearest the anode is albumin. The cathodal (*right*) boundary of the albumin fraction is not marked in the trough because there is α_1 globulin immediately cathodal to the albumin band. The α_2 fraction begins just anodal to the second distinct band cathodal to albumin. The β_1 fraction typically begins at about the midpoint of the tracing, and the end is most commonly placed prior to the most prominent β band, which is therefore in the β_2 region. The cathodal aspect of the β_2 fraction is the start of the γ fraction that appears as a diffuse staining (smear) of proteins. The major locations of proteins that can cause detectable changes in canine serum protein electrophoretic patterns are listed, but data are limited and there can be overlap (e.g., some IgG molecules can be found in the β_2 fraction, and some IgA or IgM molecules can be found in the γ fraction). The locations of transferrin and C3 within the β region are unclear. Migration can vary among individuals and methods. Many proteins have similar migrations in other mammalian species, but there are species differences.

b. Accurate quantification of protein fractions is dependent on consistent and linear dye uptake by different proteins in SPE media. The uptake of Ponceau S stain and Coomassie brilliant blue stains by albumin is at least about 1.5 times that of γ globulins in cellulose acetate,[58,59] and amido black has nonlinear absorption with changes in percentages of assessed proteins.[59] These factors contribute to inaccuracies of quantitation and to discrepancies between albumin concentrations determined by BCG and SPE methods.

c. Densitometry can also yield inaccurate results if a defective area is scanned (e.g., a staining artifact) or if band density goes beyond the linearity of the densitometer so that peak heights are not proportional to protein content (e.g., application of too much protein to the well, particularly in a sample with a monoclonal gammopathy). Protein overloading (inadequate sample dilution) may also result in reduced discrimination of adjacent bands.

3. CZE can resolve more protein fractions than AGE, but sensitivity is limited by the bore of the capillary and therefore the absorption path length, and separate fractions may merge with use of certain curve-smoothing algorithms. Because of the wavelength used for absorption, nonprotein molecules such as antibiotics or radiocontrast material may cause or contribute to the generated electrophoretic profiles.[50]

H. Interpretive considerations

1. A protein band may represent one protein (e.g., albumin) or several proteins that have migrated the same distance (common with α_2 and β globulins).
2. The same protein groups in each animal species have slight to moderate differences in migrations (e.g., CRP reportedly migrates in the β–γ region in horses but the γ region in dogs and people).[60] Also, the same proteins may migrate differently in different electrophoretic systems. Therefore, species- and method-specific reference patterns or values are needed for interpretation.
3. Equine serum has a fast-migrating α_1 globulin that yields a cathodal shoulder to the albumin peak;[61] the fast-migrating α_1 globulin is less commonly seen in canine and feline sera. The band is more prominent with hypoalbuminemic serum and with AGE rather than cellulose acetate SPE. It may also be seen with CZE.
4. With CZE, an increase in fast-migrating α_1 globulins is commonly apparent in serum from hypercholesterolemic dogs, causing either distinct peaks cathodal to the albumin peak or a prominent cathodal shoulder to the albumin peak.[62] If this α_1 globulin (likely α_1-apolipoprotein) is not excluded from the albumin fraction, the electrophoretically derived [albumin] will be falsely high and the [α_1 globulins] will be falsely low.
5. The calculated concentrations of gel electrophoretic fractions are at best estimates. A protein concentration > 0.1 g/dL may be needed before the protein can be detected by AGE (lower concentrations can be detected by CZE). The true value is usually within 0.3 g/dL of the calculated value, assuming appropriate marking of fractions. The calculated concentrations are frequently not needed to properly classify or interpret dysproteinemia patterns, as the pattern is often sufficient.
6. Protein bands on cellulose acetate or agarose gels should be examined in addition to densitometric findings because resolution of the bands is often greater than the separation seen in densitometric tracings. Band detection can be improved by (i) tipping the gel at an angle rather than looking at it perpendicularly, and (ii) viewing it with slight peripheral vision to benefit from the increased sensitivity of retinal rods.

7. Via routine AGE, globulins are most commonly grouped into 5 electrophoretic regions: α_1, α_2, β_1, β_2, and γ.[63–65] Cellulose acetate electrophoresis may resolve only four globulin fractions (α_1, α_2, β, and γ) in bovine serum.[66] With CZE, the β region may be divided into three subregions. Fractions may not separate distinctly in disease states.
8. Migration of common interferents[50,54,67]
   a. Hemolysis
      (1) Cellulose or agarose SPE: β region (free hemoglobin) and cathodal α_2 region (Hgb–Hp complexes) with possible reduction in anodal α_2 region (Hp)
      (2) CZE: β region (could be considered β_1 or β_2 depending on how one fractionates the electrophoretogram)
   b. Hyperbilirubinemia
      (1) Cellulose or agarose SPE: no change in pattern expected, but hyperbilirubinemia could cause falsely high or low [TP] in bichromatic biuret assays and thus calculated fractional concentrations would be falsely high or low
      (2) CZE: no major visual changes to pattern but may mildly increase the albumin fraction; reported alterations to other fractions were complex and not dose dependent
   c. Fbg (in plasma or incompletely clotted "serum" sample)
      (1) Cellulose or agarose SPE: β_2 region
      (2) CZE: β_2 or β–γ region with some systems (buffers) but undetected in others in which Fbg may adhere to the capillary wall

V. Immunoelectrophoresis (see Hyperproteinemia, Section II.B, Lymphoid neoplasms creating paraproteinemia)
   A. This is a method of immunologically identifying the presence of specific proteins or protein components. It can be used to identify the presence of immunoglobulin classes or subclasses, heavy chains, or light chains.
   B. Serum proteins are first separated by electrophoresis. Appropriate antibodies are added to long troughs cut parallel to the electrophoretic separation. The antibody and the electrophoretically separated serum proteins diffuse toward each other and form precipitant arcs if there is a reactive antigen (e.g., heavy chain of IgG) for the antibody (see reference for illustrations).[68]
   C. Clinically, immunoelectrophoresis is used most often to detect a monoclonal gammopathy or to identify the class (isotype, heavy chain type) of immunoglobulin in SPE-detected monoclonal gammopathies. Identifying IgA or IgM rather than IgG as the predominant immunoglobulin helps support a neoplasm (usually a plasma cell myeloma) rather than an infection (e.g., ehrlichiosis, leishmaniasis, staphylococcal pyoderma).

VI. Immunoelectrophoresis immunofixation is similar to immunoelectrophoresis in that it is a two-step process used to identify or characterize electrophoretic bands suspected to be monoclonal proteins.
   A. Step 1 is serum (or urine) electrophoresis to separate proteins into bands in replicate lanes of a gel.
   B. Step 2 is to incubate each lane with specific anti-immunoglobulin antibodies by applying separate antibody solutions to separate replicate lanes (rather than placing them in adjacent troughs as for immunoelectrophoresis). Antibodies react with their target antigens, if present, and the complexes precipitate. Other proteins are washed out and the gel is

stained. A narrow, stained band supports a monoclonal protein that can be classified based on the antibody that precipitated it. See references for illustrations.[69–71] Immunofixation is relatively rapid, sensitive (detects < 0.1 g/dL), and easy to interpret.

VII. Quantitative analysis of immunoglobulins (see Immunoglobulins section)
- A. Immunoglobulin quantitation may be used in the diagnosis of ITPI, immunoregulatory disorders, and neoplastic disease.
- B. Methods to quantitate immunoglobulin isotypes include immunoturbidimetry, nephelometry, radial immunodiffusion, and ELISA.[72] Automated immunoturbidimetric methods benefit from greater precision and faster turnaround than manual methods.

VIII. Sia euglobulin test
- A. A *euglobulin* is a protein that, unlike albumin, does not dissolve in pure water. The *Sia euglobulin test* is done by adding 1 drop of serum to demineralized H_2O; formation of a precipitate or flocculation is a positive test. When first described by Sia in 1921, the euglobulin test was a screening test for macroglobulinemia associated with visceral leishmaniasis; it was later promoted as a screening test for Waldenström macroglobulinemia, a disorder characterized by lymphoma with an IgM monoclonal gammopathy. However, analyses of human sera indicate that the positive test may result from a variety of monoclonal and polyclonal gammopathies and thus has minimal diagnostic value.[73] A modified Sia test (using weak electrolyte solutions) for macroglobulins resulted in fewer false positives but more false negatives for detecting monoclonal gammopathies.
- B. However, recognizing the precipitant property of immunoglobulins is important in two situations. First, this property can be used to separate immunoglobulins from other serum proteins; the precipitated immunoglobulins can then be dissolved in saline. Second, the precipitates can interfere with the analysis of sera or blood samples, including total protein.
  1. With marked hyperproteinemia, an analyzer dilution may be necessary to reduce the protein concentration into the analytical range. If hyperproteinemia is caused by euglobulinemia and the diluent is H_2O, the euglobulin may precipitate leading to a falsely decreased protein measurement. A saline diluent can be used instead.[74]
  2. Euglobulin precipitates may falsely increase serum phosphorus concentration (see Chapter 11), blood [Hgb] (see Chapter 3), and serum bilirubin concentration (see Chapter 13).
  3. If an analyzer indicates the presence of hemolysis or lipemia based on a water-diluted sample blank but the sample lacks visible evidence of either, a positive Sia test of the sample would support an interfering dysproteinemia.[75]

HYPERPROTEINEMIA (INCREASED TOTAL PROTEIN CONCENTRATION IN SERUM OR PLASMA)

Disorders and conditions that cause hyperproteinemia are listed in Table 7.3.

I. Hemoconcentration is a common cause of hyperproteinemia.
- A. Pathogenesis: Hyperproteinemia results from the concentration of plasma proteins caused by the loss of plasma H_2O. Plasma H_2O loss may occur with (i) fluid shifts out of the

Table 7.3. Disorders and conditions that cause hyperproteinemia[a]
Hemoconcentration*
Increased protein synthesis
Inflammatory diseases
Infection:* bacterial, viral, fungal, protozoal
Noninfectious disease:* necrosis, neoplasms, immune-mediated disease
Lymphoid neoplasms creating paraproteinemia
Plasma cell: plasma cell myeloma, extramedullary plasmacytoma
Lymphocyte: lymphoma, lymphocytic leukemia
Monoclonal gammopathy of undetermined significance (MGUS)
Polyclonal B-cell lymphocytosis of English bulldogs

* A relatively common disorder, condition, or mechanism

[a] Hyperproteinemia based on plasma [TP_{ref}] should be confirmed by a chemical test because interferents may cause false increases in [TP_{ref}].

Notes: All of these disorders or conditions may cause hyperglobulinemia, but only hemoconcentration causes concurrent hyperalbuminemia. The upper reference limits for greyhound [TP] and [globulins] are lower than for most canine breeds.

vasculature, (ii) loss from the body due to vomiting, diarrhea, impaired renal concentrating ability, sweating, or insensible loss via respiration, or (iii) decreased H_2O intake combined with normal losses.

B. If the [TP] in health were 7.0 g/dL and dehydration caused a 10 % decrease in plasma volume, the [TP] would increase to about 7.8 g/dL (7.0/0.9 = 7.78).

C. All plasma proteins are concentrated by loss of plasma H_2O; therefore, hemoconcentration results in a nonselective hyperproteinemia. Concentrations of albumin, globulins, and Fbg are proportionately increased if dehydration is the only cause of the dysproteinemia.

D. Hyperproteinemia is a poor indicator of hydration status in sick llamas and alpacas because of the high occurrence of disorders that concurrently decrease protein concentration through protein loss or protein catabolism.[76]

E. Other common laboratory findings are erythrocytosis, prerenal azotemia, and, if renal concentrating mechanisms are functional, hypersthenuria.

II. Increased protein synthesis

A. Inflammation is a common cause of increased protein synthesis but does not necessarily cause hyperproteinemia, even when there are changes in the concentrations of protein fractions.

1. Pathogenesis: Inflammation (caused by infections or other processes) stimulates the synthesis of certain globulins by hepatocytes and perhaps immunoglobulins by B lymphocytes. Several cytokines, especially IL-6, alter protein synthesis in, or protein release from, hepatocytes.[77,78] Cytokines primarily regulate transcription (either upregulate or downregulate) to alter protein production to produce these changes.

a. Increased concentrations of the PAPPs (see General Concepts for Total Protein, Albumin, and Globulins, Section II.C)

(1) Production of these proteins may increase within minutes and may persist as long as an inflammatory process is present (from acute to chronic

inflammatory disease). Increased plasma or serum concentrations may be seen by 2 d after the onset of inflammation.

(2) Individually, Fbg and Hp concentrations can increase enough to increase [TP] noticeably. [Fbg] may increase plasma [TP] by increments of 0.3 g/dL up to perhaps 1.5 g/dL, and [Hp] may increase serum or plasma [TP] by increments of 0.3–0.9 g/dL. The concentrations of other PAPPs are relatively much less in health, and thus a marked increase in such an individual protein concentration adds little to the [TP]. For example, [CRP] may increase from 0.001 g/dL to 0.05 g/dL, and [SAA] may increase from 0.0002 g/dL to 0.1 g/dL;[22] units of g/dL (instead of mg/dL) are used to illustrate the insignificant impact on measured [TP].

b. Concurrent decreases in concentrations of NAPPs

(1) NAPP production by hepatocytes decreases during inflammation.

(2) Hypoalbuminemia (mild to moderate) does not develop from this mechanism alone until inflammation has persisted for days to weeks. It may be detected within a few days in cats and dogs, but weeks are required for detection in larger species with longer albumin half-lives (see Hypoalbuminemia section). Because of inter-individual variation, this negative acute phase response may be more readily detected by comparing [albumin] to a patient's own baseline (homeostatic setpoint) instead of to a population-based RI.

(3) The plasma half-life of transferrin is not firmly established in domestic species but is typically stated to be 8–10 d in heathy people. In experimentally-induced sterile inflammation in dogs, total iron binding capacity (transferrin) reached a nadir at about 17 d.[79]

c. Increased concentrations of immunoglobulins (mostly IgG): Increased synthesis of a variety of immunoglobulins by many clones of B lymphocytes produces a *polyclonal gammopathy,* which is typically characterized by SPE as a broad band or smear of protein that appears as a low, broad hump on a densitometer tracing. A detectable increase in serum [globulins] generally requires 1–3 wk from the onset of inflammation. The magnitude of increase in [immunoglobulin] can be mild (< 1.0 g/dL) to marked (> 4.0 g/dL)

2. Expected dysproteinemia patterns

a. Acute-phase response: hyperproteinemia caused by acute inflammation lasting 2–7 d

(1) Mild hyperproteinemia characterized by hyperglobulinemia (increased α_1 and/or α_2 globulins and hyperfibrinogenemia)

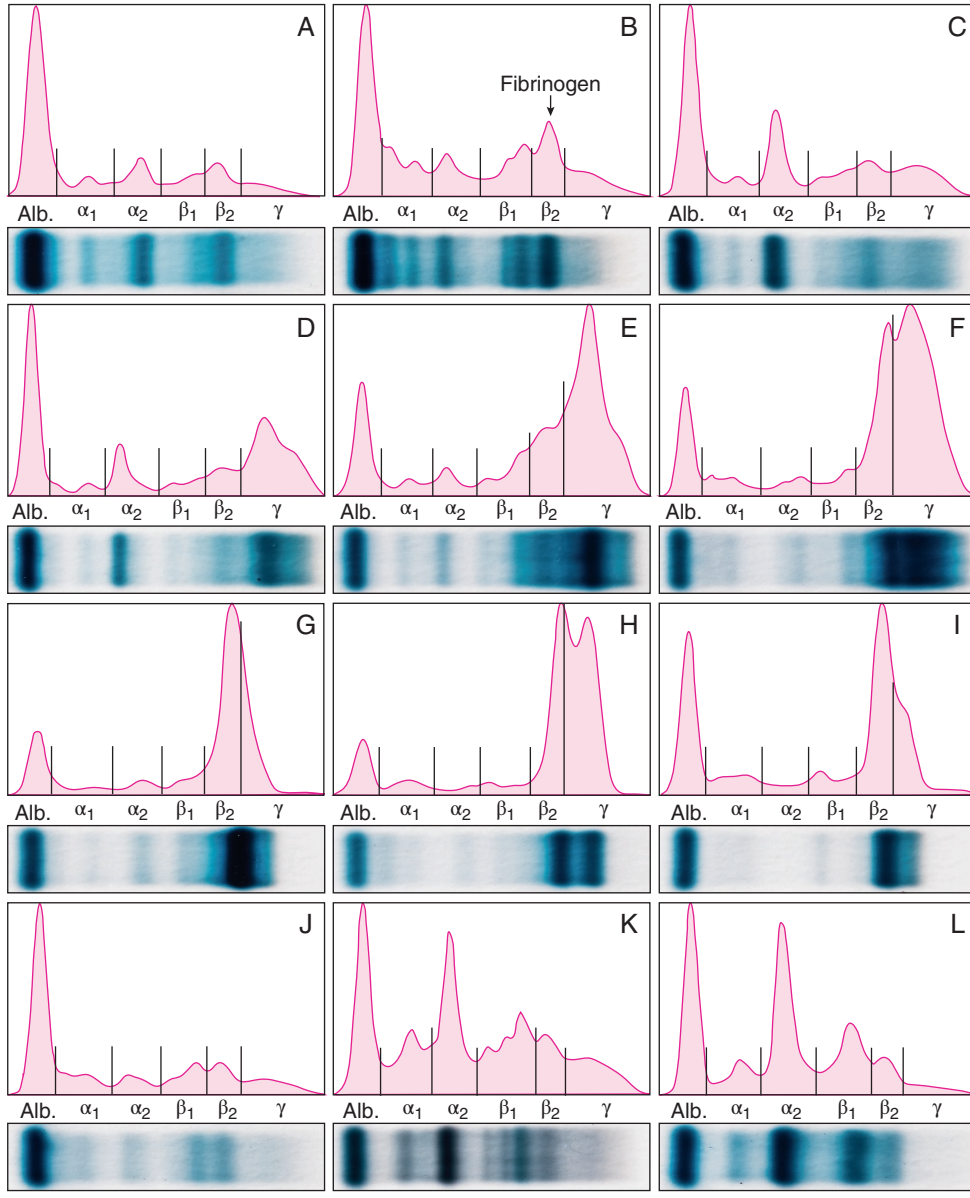
(2) Possibly mild hypoalbuminemia or low-normal serum or plasma albumin concentration

b. Delayed response: hyperproteinemia caused by inflammation lasting more than 7 d

(1) Hyperproteinemia may be mild to marked (e.g., 10 g/dL) and is characterized by hyperglobulinemia from increased immunoglobulins (mostly γ globulins) forming a polyclonal gammopathy (Figure 7.4C–F, Figure 7.5C, D, J); PAPPs may also be increased (Figure 7.4C and D; Figure 7.5C and D).

(2) Mild to moderate hypoalbuminemia may be present.

(3) A polyclonal gammopathy with a restricted migration has been called an *oligoclonal gammopathy* or *restricted polyclonal gammopathy* (Figure 7.4D and E). *Oligoclonal* means having few clones, therefore yielding few different immunoglobulins and few protein bands rather than a broad smear of many



Canine		A	B	C	D	E	F	G	H	I	J	K	L	Ref. Int.
TP (biuret)	g/dL	6.7	6.9	7.5	8.0	11.3	11.9	11.8	10.3	10.3	4.2	5.9	5.3	5.4–6.9
Alb. (BCG)	g/dL	3.8	3.2	3.3	2.8	1.8	1.9	1.8	1.1	3.0	2.2	2.2	2.2	3.2–4.1
Globulins	g/dL	2.9	3.7	4.2	5.2	9.5	10.0	10.0	9.2	7.3	2.0	3.7	3.1	1.9–3.2
Alb. (SPE)	g/dL	3.4	2.4	2.8	2.7	1.7	1.4	1.9	1.0	2.8	1.9	1.4	1.5	NA*

* NA - not available

Figure 7.4. Canine serum protein electrophoresis (SPE) and plasma protein electrophoresis (frame B). SPE is most valuable when gel band patterns and densitometer tracing patterns are evaluated concurrently and with knowledge of the sample's [TP] by chemical analysis. The table below the SPE images provides the protein concentrations in the samples and canine reference intervals for the biuret assay (TP), BCG assay (albumin), and the corresponding

Figure 7.4. *continued*
concentrations of globulins. Note: for SPE densitometer tracings, the darkest band in the stained gel produces the tallest portion of the tracing; the heights of the curves for all other bands are relative to the darkest band.
A. Healthy: The densitometer tracing is within expected results for healthy dogs and is provided as a reference pattern; minor variations in the distribution of protein fractions would be found in other healthy dogs. The marking of SPE fractions in frame A was used as a basis to mark SPE fractions in frames B–L, with minor adjustments for apparent alterations in migration that might relate to individual dog variations, minor procedural variations, or altered protein proportions and concentrations.
B. Mild inflammatory dysproteinemia (plasma sample): Because fibrinogen forms a prominent band in the β_2 fraction, a region where paraprotein bands commonly occur, serum rather than plasma is the sample of choice for electrophoresis.
C. Mild inflammatory hyperproteinemia (selective pattern): The hyperglobulinemia is characterized by increased α_2 and γ fractions. The increased α_2 region is due to increased concentrations of Hp or α_2-macroglobulin (PAPPs). The increased γ region is broad-based and is thus due to a polyclonal gammopathy (mostly IgG). Note the 0.5 g/dL difference in albumin concentrations.[a]
D. Inflammatory hyperproteinemia (selective pattern): A polyclonal gammopathy (mostly IgG) is the major contributor to the hyperglobulinemia; the dense anodal band in the γ region suggests an oligoclonal gammopathy within the polyclonal gammopathy.
E. Inflammatory hyperproteinemia (selective pattern): The hyperglobulinemia is characterized by increased β_1-, β_2-, and γ-globulin concentrations. The dense band and peak in the γ fraction might suggest a monoclonal or oligoclonal gammopathy, but the diffuse cathodal (*toward the right*) γ fraction is not expected in a neoplastic monoclonal gammopathy, because immunoglobulin production by nonneoplastic lymphoid cells is commonly diminished.
F. Inflammatory hyperproteinemia (selective pattern): The hyperglobulinemia is characterized by increased β_2- and γ-globulin concentrations. The diffuse protein smear across the γ region is indicative of a polyclonal gammopathy.
G. Paraproteinemia with monoclonal gammopathy (selective pattern): The hyperglobulinemia is characterized by a distinct dense band and increased protein concentration in the β_2-γ region. As is expected with marked paraproteinemia, electrophoresis of diluted serum (not shown) produced a similar pattern but with a narrower β_2 band and poorly detectable α_1, α_2, and β_1 bands. The reason for stained proteins on both sides of the β_2 band was not determined. The absence of detected proteins in the cathodal portion of the γ fraction, concurrent with the β_2-γ hyperglobulinemia, is indicative of a paraproteinemia; polyclonal production of IgG is expected in health and inflammatory hyperproteinemia, whereas it is diminished with paraproteinemia. The SPE-determined [albumin] was greater than the BCG [albumin]; this may have occurred if the density of the paraprotein band exceeded the linearity of the densitometer, thus underestimating the paraprotein concentration and overestimating the concentrations of all other protein fractions.
H. Paraproteinemia with a monoclonal or biclonal gammopathy[b] (selective pattern) (diluted serum):[c] The hyperglobulinemia is characterized by two distinct electrophoretic bands, one in the β_2-γ region and one in the γ fraction; the two bands were not distinct with electrophoresis of undiluted serum. The concurrent absence of detected proteins in the cathodal portion of the γ fraction is typical of a paraproteinemia.
I. Paraproteinemia with a monoclonal or biclonal gammopathy[b] (selective pattern) (diluted serum)[c]: The hyperglobulinemia is characterized by increased β_2- and γ-globulin concentrations, a dense band in the β_2 region, and an adjacent, paler band in the γ region that was not apparent in the agarose gel pattern of undiluted serum (it appeared as part of one broad band) and appears as a shoulder of the β_2 peak. The paucity of detected proteins in the cathodal portion of the γ fraction is typical of a paraproteinemia. Sample dilution lowered the protein concentrations enough that bands between albumin and β_2 globulins are barely visible.
J. Panhypoproteinemia (nonselective pattern): Protein concentrations are decreased proportionately or nearly so, as indicated by a tracing similar to that in health (frame A). Causes of panhypoproteinemia include acute blood loss, maldigestive and malabsorptive disorders, early third-space loss (after intravascular fluid replacement), starvation, cachexia, and occasionally hepatic failure.

continued on next page

different proteins. In human medicine, oligoclonal gammopathies can be differentiated from monoclonal gammopathies by increases in κ and λ light chains rather than by just one of the light chain types. The "monoclonal" gammopathies that some authors have described in animals with infectious diseases (e.g., ehrlichiosis and leishmaniasis, rarely chronic pyoderma and heartworm disease) may have been restricted polyclonal gammopathies.[80–87] They were considered monoclonal gammopathies because of the relatively narrow peak in an electrophoretic densitometry tracing, and the authors established that the gammopathy was primarily caused by one class of immunoglobulin (i.e., IgG) via immunoelectrophoresis or radial immunodiffusion. However, the gammopathy could include more than one immunoglobulin subclass and thus not be monoclonal, and light-chain analyses were not done to help support clonality.

(4) A biclonal pattern (electrophoretic α_2 and β_1 spikes) caused by an increased [IgG_3] was found in a horse. The gammopathy disappeared after the horse was treated for a strongyle infection.[86] IgG_3 is also referred to as IgG(T).

c. A monoclonal expansion of T lymphocytes has been found in dogs infected with *Ehrlichia canis* that concurrently had gammopathies with narrow spikes in electrophoretic densitometry tracings.[87] We are not aware of cases in which inflammation caused a monoclonal gammopathy from clonal expansion of B lymphocytes or plasma cells.

3. Other laboratory data that may develop with inflammatory hyperproteinemia
   a. Anemia of inflammatory disease if inflammation persists
   b. Inflammatory neutrophilia with or without a left shift or toxic change
   c. Inflammatory lymphocytosis or lymphopenia
   d. Inflammatory monocytosis
4. Concurrent pathologic processes may complicate interpretation of the protein data in inflammatory states. For example, there can be increased Fbg production because of inflammation concurrent with increased Fbg consumption because of intravascular coagulation. Hemoconcentration may occur concurrently with protein-losing states.

B. Lymphoid neoplasms creating paraproteinemia

Figure 7.4. *continued*

K. Dysproteinemia of a protein-losing nephropathy (selective pattern): The prominent α_2 band and hyperglobulinemia suggest that an α_2 globulin (most likely α_2-macroglobulin) is being retained by the kidneys more than the other globulins and/or there is increased production of one or more α_2 globulins. However, hypoalbuminemia is causing all the globulin fractions to appear increased by densitometry because the fractions' heights are relative to the albumin peak. Note the 0.8 g/dL difference in albumin concentrations.[a]

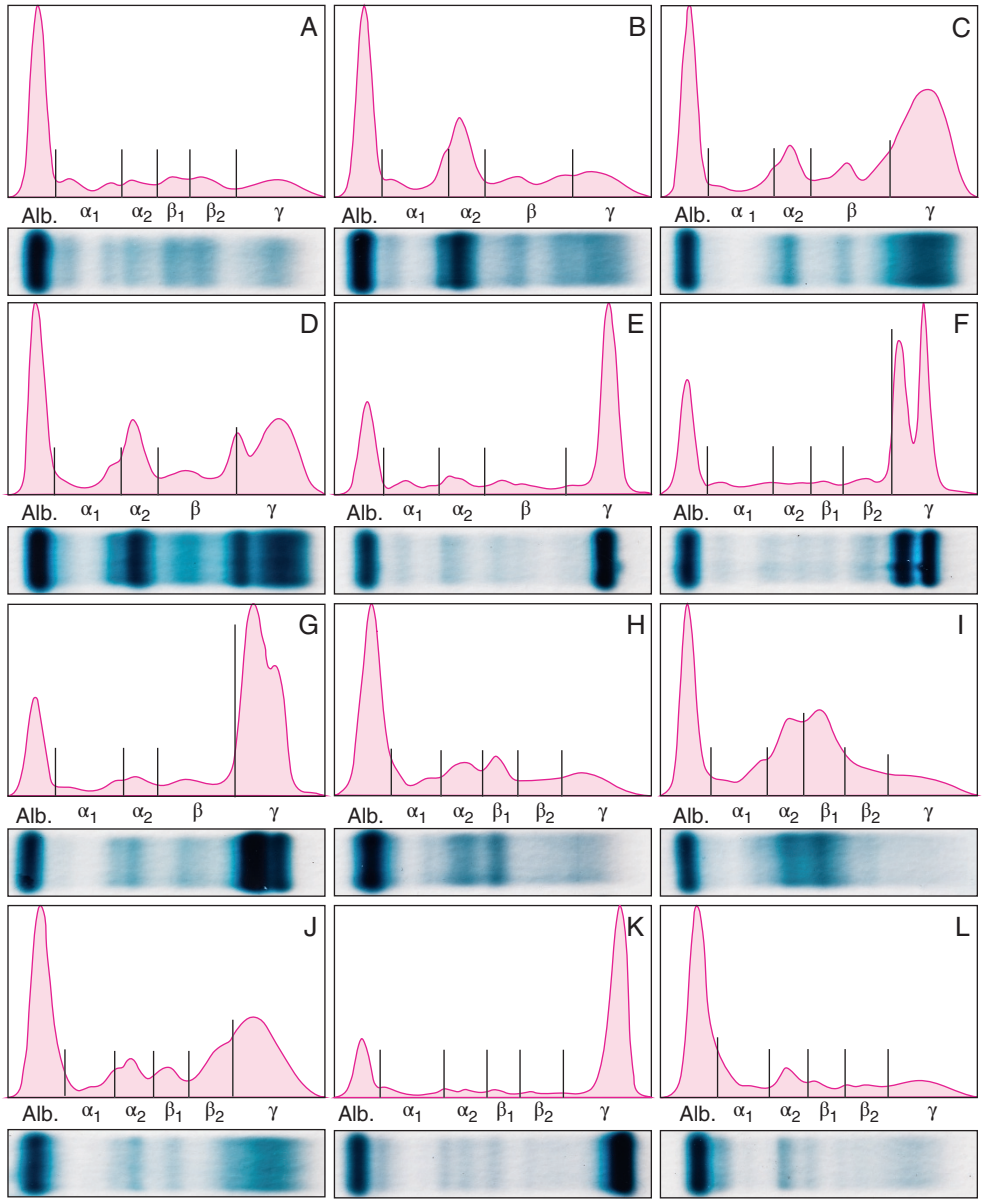
L. Hypoproteinemia of a protein-losing nephropathy (selective pattern): a variation of the findings in frame K, with a more prominent α_2 band and less γ globulin. Note the 0.7 g/dL difference in albumin concentrations.[a] Fraction divisions (β_1-β_2 and β_2-γ) were substantially adjusted from those in frame A to separate distinct β bands.

[a] A difference greater than 0.3 g/dL is common when there is an increase in α_2 globulins compared to albumin; the BCG dye binds to some α_2 globulins, thereby increasing the measured [albumin].

[b] The biclonal pattern could truly be a biclonal gammopathy, but also could be a monoclonal gammopathy with a second band created by immunoglobulin degradation or immunoglobin complexing with itself or another protein. Immunofixation or immunoelectrophoresis is needed to determine which immunoglobin class(es) is (are) present.

[c] 1 part serum to 1 part saline; some systems routinely require greater serum dilution

1. Pathogenesis: Certain lymphoid neoplasms may produce large quantities of an immunoglobulin, typically from one clone of neoplastic cells. The single clone of lymphocytes produces an electrophoretically, structurally, and antigenically homogeneous immunoglobulin or immunoglobulin subunit.
   a. The resulting dysproteinemia is called a *monoclonal gammopathy* or *paraproteinemia*.
   b. An intact monoclonal immunoglobulin in most mammalian species consists of a single heavy chain class (e.g., IgM) and subclass (e.g., IgG_1 or IgG_2) and two light chains of the same type (either κ or λ but not both) (Figure 7.2). Monoclonal gammopathies have not been reported in camelids.
   c. Monoclonal immunoglobulin subunits may be free light chains,[88,89] heavy chains only,[90] or abnormal fragments.[91]
2. Immunoglobulin proteins produced by lymphoid neoplasms are sometimes called *M proteins* (for monoclonal proteins). *M protein* has also been used as an abbreviation for myeloma proteins or macroglobulin, and thus one must interpret "M protein" in context. The proteins that are produced by neoplastic lymphoid cells and accumulate in plasma, urine, or tissues are *paraproteins*.
3. Types of lymphoid neoplasms that may cause a gammopathy
   a. Plasma cell neoplasm: most often a plasma cell myeloma (proliferating in the bone marrow) but also some extramedullary plasmacytomas
   b. B-lymphocyte lymphoma or B-lymphocytic leukemia
4. Expected dysproteinemia pattern (see the following section for SPE details)
   a. When clinical disease becomes apparent, there is often moderate to marked hyperproteinemia and hyperglobulinemia caused by the monoclonal gammopathy. However, a lymphocyte or plasma cell neoplasm may produce only a mild monoclonal gammopathy without hyperglobulinemia[92–94] or, uncommonly, be oligosecretory or nonsecretory with little or no evidence of secreted protein in plasma.[95]
   b. Mild to moderate hypoalbuminemia may be present. Possible causes include decreased albumin synthesis due to inflammatory cytokines, decreased synthesis in response to increased oncotic pressure,[96–98] or a concurrent pathologic process.
5. Serum protein electrophoresis findings
   a. A monoclonal protein typically forms a narrow protein band and corresponding densitometer tracing peak (i.e., resembling an albumin band/peak) in the β- or γ-globulin fractions (Figure 7.4G; Figure 7.5E and K). However, a monoclonal protein can be present in low concentrations that do not cause hyperglobulinemia or definite abnormalities in routine AGE.
      (1) Most IgG monoclonal proteins migrate in the γ fraction or at the β–γ junction, but some migrate more anodally in the β region,[99] and they may migrate to the α_2 fraction in horses.[100] IgM and IgA monoclonal antibodies typically migrate at the β–γ junction or in the β-globulin fraction, but may migrate to the γ fraction in horses.[100] Variation in the marking of electrophoretogram fractions causes inconsistent classification of protein fractions in the β and γ regions.
      (2) A marked hyperglobulinemia characterized as a β gammopathy was initially considered to mimic a monoclonal gammopathy in a dog with suspected IgG4-related disease, a poorly understood immune-mediated condition, but the β band was broad and hypogammaglobulinemia was absent.[101] Besides hyperglobulinemia, the dog had eosinophilia, increased serum [IgE], and moderate plasmacytosis in lymph nodes and bone marrow. Similar mild to marked β hyperglobulinemias occur in dogs with angiostrongylosis.[102]



Feline		A	B	C	D	E	F	G	Ref. Int.
TP (biuret)	g/dL	6.4	7.2	9.8	8.5	11.5	12.8	14.7	6.2–8.5
Alb. (BCG)	g/dL	3.5	3.2	2.8	2.8	2.2	2.2	2.1	3.2–4.5
Globulins	g/dL	2.9	4.0	7.0	5.7	9.3	10.6	12.6	2.6–5.0
Alb. (SPE)	g/dL	3.1	2.7	2.5	2.7	2.7	2.9	2.5	NA*

Equine		H	I	J	K	L	Ref. Int.
TP (biuret)	g/dL	6.6	8.9	8.6	13.2	5.0	5.8–7.5
Alb. (BCG)	g/dL	3.3	2.6	2.9	1.6	2.9	2.9–3.7
Globulins	g/dL	3.3	6.3	5.7	11.6	2.1	2.6–4.2
Alb. (SPE)	g/dL	3.2	2.5	3.0	2.4	2.7	NA*

* NA - not available

Figure 7.5. Feline and equine serum protein electrophoresis (SPE). SPE is most valuable when gel band patterns and densitometer tracing patterns are evaluated concurrently and with knowledge of the sample's [TP] by chemical analysis. The table below the SPE images provides the protein concentrations in the samples and reference intervals for the biuret assay (TP), BCG assay (albumin), and the corresponding concentrations of globulins. Note: for SPE densitometer tracings, the darkest band in the stained gel produces the tallest portion of the tracing; the heights of the curves for all other bands are relative to the darkest band.

A. Cat, healthy: The densitometer tracing is within expected results for healthy cats and is provided as a reference pattern; minor variations in the distribution of protein fractions would be found in other healthy cats. The cathodal (*toward the right*) boundary of the albumin fraction separates albumin from an anodal α_1 band. The α_2-globulin fraction encompasses the third definitive band cathodal to albumin, ending just anodal to the midpoint of the tracing. Not all healthy cats have the definitive β_1 and β_2 bands as seen here. The γ-globulin fraction appears as a diffuse staining (smear) of proteins. The marking of SPE fractions in this cat was used as a template to mark SPE fractions in frames B–G, with minor adjustments for apparent alterations in migration that might relate to individual cat variations, minor procedural variations, or altered protein proportions and concentrations. β subdivisions are not always apparent in cats and were not marked for every cat.

B. Cat, mild inflammatory dysproteinemia (selective pattern): The increased density of α_2 globulins is due to increased concentrations of Hp or α_2-macroglobulin (PAPPs). There might be a slight increase in γ globulins. Note the 0.5 g/dL difference in albumin concentrations.[a]

C. Cat, inflammatory hyperproteinemia (selective pattern): The hyperglobulinemia is characterized by an increase in proteins throughout the γ region and extending into the β_2 region, thus indicating a polyclonal gammopathy (mostly IgG). The α_2-globulin concentration might be mildly increased based on the density of the band, but the height of the corresponding tracing hump is increased partly because of the hypoalbuminemia (a relative increase).

D. Cat, inflammatory hyperproteinemia (selective pattern): The hyperglobulinemia is characterized by increased γ-globulin (polyclonal gammopathy) and increased α_2-globulin and cathodal β-globulin concentrations. The increased prominence of α_2 globulins is due to increased concentrations of Hp or α_2-macroglobulin (PAPPs). The distinct band at the β-γ-globulin junction is unusual in inflammatory states and its cause was not determined.

E. Cat, paraproteinemia with monoclonal gammopathy (selective pattern) (diluted serum)[b]: The distinct γ band and corresponding narrow-based γ-globulin peak supports a monoclonal gammopathy (probably IgG). The other fractions in the densitometer tracing are low because they are relative to the γ-globulin peak; the albumin peak is also low because of hypoalbuminemia.

F. Cat, paraproteinemia with a monoclonal or biclonal gammopathy[c] (selective pattern) (diluted serum)[b]: The hyperglobulinemia is characterized by two distinct, dense bands causing increased β_2-γ- and γ-globulin concentrations. The paucity of detected proteins in the cathodal portion of the γ fraction is typical of a paraproteinemia; immunoglobulins other than the paraproteins are frequently decreased. The rest of the densitometer tracing is low because it is relative to the γ-globulin peak, and the albumin peak is also low because of hypoalbuminemia.

G. Cat, paraproteinemia with monoclonal or biclonal gammopathy[c] (selective pattern) (diluted serum)[b]: The hyperglobulinemia is characterized by two dense juxtaposed bands in the γ region, thus causing an increased γ-globulin concentration. Greater dilutions of the serum might have allowed better visualization of two distinct bands. See the frame F caption above regarding the appearance of the rest of the tracing.

H. Horse, healthy: The densitometer tracing is within expected results for healthy horses and is provided as a reference pattern; minor variations in the distribution of protein fractions would be found in other healthy horses. The cathodal (*toward the right*) boundary of the albumin fraction excludes the most anodal-migrating α_1 globulins. The α_2 fraction encompasses the band immediately anodal to the midpoint of the tracing. The β_1 and β_2 fractions may or may not have definitive bands in healthy horses, and there may not be a distinct β_2-γ junction. The markings of the SPE fractions in this frame were used as a template to mark fractions in frames I–L, with minor adjustments for apparent alterations in migration that might relate to individual horse variations, minor procedural variations, or altered protein proportions and concentrations.

I. Horse, inflammatory hyperproteinemia (selective pattern): The hyperglobulinemia is mostly the result of increased α_2- and β_1-globulin concentrations (note increased density of bands), probably reflecting increased

continued on next page

(3) Some horses with plasma cell myelomas generate a monoclonal IgG band/peak in the α_2-globulin fraction; this is supportive of IgG_3 or IgG_5, which together are also called IgG(T).[100]

(4) Atypical electrophoretic migrations of paraproteins may be caused by protein degradation, immunoglobulin binding to other proteins, formation of immunoglobulin complexes, multiple clones (e.g., biclonal gammopathy), or production of incomplete immunoglobulins (e.g., light chains, heavy chains, or abnormal fragments) (Figure 7.4H and I; Figure 7.5F and G).[93]

(a) Immunoglobulin complexes: Complexes of whole immunoglobulins or immunoglobulin subunits may migrate differently from monomers, thus yielding separate bands/peaks for monomers, dimers, trimers, or other multimers.[88,103] Treatment of the serum with a reducing agent prior to electrophoresis may yield a single monoclonal band/peak because of dissociation of the complexes.[104]

(b) Multiple clones: Double bands/peaks may also be caused by two different clones of immunoglobulin-producing plasma cells.[68,105]

b. When a marked monoclonal gammopathy is present, concentrations of other immunoglobulins frequently are decreased; this secondary hypogammaglobulinemia may contribute to impaired immunity.[92]

Figure 7.5. *continued*

PAPPs (immunofixation needed to identify proteins). The height of the curve throughout the globulin regions is also partially due to the mild hypoalbuminemia, which might relate to albumin being a negative acute-phase protein.

J. Horse, inflammatory hyperproteinemia (selective pattern): The hyperglobulinemia is characterized by increased β_2 and diffuse γ globulins, reflecting a polyclonal gammopathy (mostly IgG).

K. Horse, paraproteinemia with monoclonal gammopathy (selective pattern) (diluted serum)[b]: The distinct γ band and corresponding narrow-based, cathodal γ peak supports a monoclonal gammopathy (probably IgG). Faint staining anodal to the dark γ band might represent protein deterioration or immunoglobulin binding to other proteins. The rest of the densitometer tracing curve is low because it is relative to the γ peak, and, for albumin, because of hypoalbuminemia. The SPE-determined [albumin] was greater than the BCG [albumin]; this may have occurred if the density of the paraprotein band exceeded the linearity of the densitometer, thus underestimating the paraprotein concentration and overestimating the concentrations of all other protein fractions. Because of the hypoalbuminemia, the anodal band in the α_1 fraction is more distinct than in frame H.

L. Horse, panhypoproteinemia (nonselective pattern): Protein concentrations are decreased proportionately, or nearly so. The anodal band in the α_1 fraction is more apparent than in health because of hypoalbuminemia. Causes of panhypoproteinemia include acute blood loss, maldigestive and malabsorptive disorders, early third-space loss (after intravascular fluid replacement), starvation, cachexia, and occasionally hepatic failure.

[a] A difference greater than 0.3 g/dL is common when there is a relative increase in α_2 globulins compared to albumin; the BCG dye binds to some α_2 globulins, thus increasing the measured [albumin].

[b] 1 part serum to 1 part saline; some systems routinely require greater serum dilution

[c] The biclonal pattern could truly be a biclonal gammopathy, but also could be a monoclonal gammopathy with a second band created by immunoglobulin degradation or immunoglobin complexing with itself or another protein. Immunofixation or immunoelectrophoresis is needed to determine which immunoglobin class(es) is (are) present.

c. Although a narrow protein band/peak in the γ-globulin region is frequently stated to represent a monoclonal gammopathy, further testing is required to definitively differentiate a monoclonal gammopathy from an oligoclonal gammopathy caused by an immune response (see preceding Hyperproteinemia Section II.A.2.b(3)). Compared to cellulose acetate methods, AGE or high-resolution electrophoretic methods such as CZE improve detection of protein bands; what appears to be one band on cellulose acetate may be seen as two bands with the other methods.[105] However, the presence of a single band still does not confirm the presence of a monoclonal gammopathy.[91] When the gammopathy appears to be monoclonal, sometimes diluting the sample with saline prior to electrophoresis allows visualization of more than one band.

6. Further testing to characterize a suspected monoclonal gammopathy
   a. Immunoelectrophoresis
      (1) If a monoclonal gammopathy is suspected, then immunoelectrophoresis with species-appropriate anti-IgG (including subclasses), anti-IgM, anti-IgA, anti-(κ chain), or anti-(λ chain) antibodies is needed to differentiate monoclonal and polyclonal gammopathies by protein analysis.
      (2) If there is a monoclonal gammopathy, then the increased concentration of one heavy-chain class (or subclass) is associated with either κ or λ chains because a lymphocyte clone produces either κ or λ chains, not both.[106]
      (3) A potential monoclonal gammopathy associated with canine ehrlichiosis was investigated by analysis of urinary light chains with an immunoprecipitation assay designed for human light chains.[107] If these antibodies had appropriate specificity for canine κ and λ light chains (they may not have), then the finding of κ and λ light chains in a 2:1 ratio suggests that the gammopathy was polyclonal.
   b. Immunofixation electrophoresis is reported to have greater analytical sensitivity than immunoelectrophoresis, so it can be useful to identify small monoclonal protein bands.[69–71] Like immunoelectrophoresis, complete testing requires monospecific and species-appropriate antisera to IgG, IgM, IgA, and free and bound κ or λ chains.
   c. Serum protein electrophoresis with densitometry may be used to estimate the area under the curve attributable to a monoclonal immunoglobulin, but the presence of other proteins migrating in the same region causes substantial error when the paraproteinemia is not marked.[108] This method may be useful for monitoring response to therapy.
   d. RID assays for [IgM] and [IgA] may be used to support a monoclonal gammopathy conclusion because high concentrations of IgM or IgA are not expected in inflammatory states. However, as for immunoelectrophoresis, RID assays for total [IgG] cannot be used to differentiate an IgG polyclonal gammopathy from an IgG monoclonal gammopathy because the IgG gammopathy may contain more than one subclass of IgG.
   e. Identification of light chains
      (1) Antibodies to human light chains can specifically differentiate κ and λ subtypes and whether they are free or bound to heavy chains. Similar panels of species-specific reagents are not currently available for domestic species. Available antibodies for human light chains can detect many light-chain proteins in dogs and cats, but they may not detect them all, bind strongly, or maintain κ and λ chain specificity.[89] Using such antibodies to differentiate canine κ and λ light chains by immunofixation did not align with identification based on liquid chromatography and tandem mass spectrometry.[89] Also, an

immunoturbidometric method using antibodies to human free κ and λ light chains lacked diagnostic utility for canine serum samples.[106]

(2) The reported marked predominance of canine[109] and feline[110] plasmacytomas with a λ-light chain phenotype and the λ:κ ratio of about 9:1 for plasma cells in lymphoid organs of healthy dogs, cats, horses, and cattle[111] might be accurate or reflect inappropriate detection by anti-(human light chain) antibodies. Therefore, dominance of λ light chains (when antibodies to human light chains are used) in tissue or serum could potentially be either a polyclonal or monoclonal proliferation in dogs, cats, horses, and cattle. Immunofixation with antibodies for human light chains supported the presence of a κ-light chain monoclonal gammopathy in a horse with a plasma cell neoplasm.[112]

(3) Considering the need for species-specific antisera for the analysis of many proteins and polypeptides, immunoglobulin characterization would be strengthened by developing species-specific reagents or establishing the degree of cross-reactivity of anti-(human light chain) antibodies with domestic mammal light chains, especially with κ light chains.

(4) Although veterinary immunoassays are currently problematic, assessments of total λ and κ serum free light chains as well as λ:κ ratios are recommended in the diagnosis and monitoring of monoclonal gammopathies.[89,113] Mass spectrometry may be helpful. In one study, λ:κ ratios were determined by immunofixation with antibodies for human light chains in sera from 10 dogs with secretory plasma cell neoplasms. Five canine sera contained predominantly λ light chains and three sera were κ predominant. A canine-specific liquid chromatography tandem mass spectrometry method supported these findings and identified two additional κ-predominant sera. In addition, the mass spectrometry method yielded significant differences in serum λ:κ ratios between dogs with secretory plasma cell neoplasms and dogs with inflammatory disease or dogs lacking SPE evidence of paraproteinemia or inflammation.[106]

7. Associated laboratory or clinical problems
   a. Bence Jones proteinuria (Figure 7.6) (Bence Jones proteins = light chains of immunoglobulins in the urine) (see Chapter 8)
   b. Hyperviscosity syndrome[114]

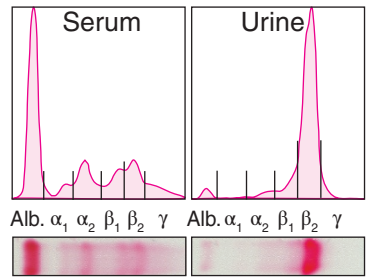


Figure 7.6. Serum and urine protein electrophoresis for dog with a Bence Jones proteinuria. The dog was panhypoproteinemic ([TP] = 3.5 g/dL) and had multicentric lymphoma with Mott cell differentiation. The dog's proteinuria (530 mg/dL with USG_{ref} = 1.068) contained a prominent β_2 globulin supportive of a Bence Jones protein. A distinct band in the urine but not in serum suggests a low-M_r neoplastic cell product (e.g., light chains) that was rapidly cleared from plasma through the glomerular filtration barrier. However, paraproteinemia is often present with Bence Jones proteinuria. Cellulose acetate electrophoresis with Ponceau S staining.

(1) High immunoglobulin concentrations may cause plasma to become viscous, particularly with larger molecules (i.e., IgM, polymerized IgA, or polymerized IgG); paraprotein concentration alone does not predict the degree of viscosity, but viscosity increases logarithmically with it.[115] Therefore, relatively small reductions in plasma [TP] by phlebotomy can result in significant reductions in viscosity. In veterinary medicine, hyperviscosity syndrome has been reported with high concentrations of IgA, IgG, or IgM.[116–118]

(2) Viscous plasma leads to sluggish blood flow in capillaries and causes poor perfusion and thus tissue hypoxia (stagnant hypoxia). Major tissues affected include brain, eyes, and kidneys. Hyperviscosity concurrent with expanded blood volume can lead to congestive heart failure by increasing workload on the heart.[119]

(3) High plasma [TP] also causes abnormalities in primary and secondary hemostasis that may lead to bleeding.[120]

c. Hypercalcemia may occur with some lymphoproliferative neoplasms (see Chapter 11).

d. Animals can develop systemic AL amyloidosis. AL-type amyloid is composed of immunoglobulin light chains. A horse with multiple myeloma was found to have systemic AL amyloidosis with an amyloid that reacted with anti-(human λ chain) antibodies.[121] The horse had hypoglobulinemia, which suggests that the neoplastic plasma cells were producing λ chains and not intact immunoglobulins (λ chains would be excreted in urine). AL amyloidosis was also diagnosed in a cat that had an extramedullary plasmacytoma.[122] Peptides within the amyloid had amino acid sequences very similar to sequences of human λ chains.

e. High concentrations of paraproteins can interfere with other laboratory test results, often because they precipitate during testing and alter absorption readings in photometric or turbidometric assays.[74] Such effects are method dependent and should be considered when patients with paraproteinemia have unexpected laboratory results.

(1) When measured by indirect potentiometry (common) or flame photometry (rare), $[Na^+]$, $[Cl^-]$, and $[K^+]$ are falsely lowered with increasing protein concentration (see Chapter 9). Pseudohyponatremia, pseudohypochloremia, and potentially pseudohypokalemia may be present with any marked hyperproteinemia.

(2) A marked false increase in hemoglobin concentration was associated with precipitation of an IgM paraprotein by a reagent containing quaternary ammonium salts to lyse cells.[123]

(3) Marked pseudohyperphosphatemia occurred in a dog with IgM paraproteinemia.[124] This was attributable to IgM precipitation in a colorimetric assay; it did not occur with a slide assay that separates proteins from plasma prior to the phosphorus reaction.

(4) Increased sample viscosity can interfere with sample aspiration into analyzers, thus potentially affecting a variety of results.

(5) As rarely reported in people, a dog with paraproteinemia had a false unmeasurably low [glucose] when glucose was measured with a colorimetric hexokinase method, but it was normoglycemic based on a point-of-care coulometric analyzer.[125] The mechanism was not determined. Paraproteinemia in this dog also appeared to cause false increases in some infectious disease titers with certain methods including a SNAP test.

8. Amplification of variable regions of immunoglobulin genes by PCR is used to identify clonal populations that may support a diagnosis of a B-lymphocyte neoplasm.[126] However, analysis of small samples from reactive lymphoid tissue may yield what appears to be a monoclonal proliferation because of reactive clonal expansion within a germinal center or because of nonspecific amplification of small amounts of DNA. Amplification may produce one or more distinct peaks that suggest clonality, but if the result is not repeatable, this finding is considered *pseudoclonality*.

C. MGUS
   1. In people, MGUS is considered a premalignant condition in which a clonal population of plasma cells has not expanded enough to meet criteria for malignancy; however, not all patients with MGUS develop a malignancy. Although considered a benign condition, patients with MGUS can have pathologic changes associated with the gammopathy.[127]
   2. Monoclonal gammopathy in the absence of lymphoid neoplasms or infectious disorders is rarely reported in domestic mammals.
      a. In one case report, a dog had primary hyperparathyroidism with a β-region IgG monoclonal or oligoclonal gammopathy that was markedly attenuated 28 d after surgical removal of the adenoma.[128]
      b. In a review of cats with plasma cell myelomas, one cat had been diagnosed with MGUS nine years before developing clinical signs and laboratory abnormalities diagnostic of a myeloma.[129]

D. Idiopathic polyclonal B-cell lymphocytosis of English bulldogs:[130] This idiopathic syndrome is characterized by a nonclonal expansion of B lymphocytes that is commonly associated with splenomegaly or splenic masses, hyperglobulinemia that can be marked, a restricted IgA ± IgM polyclonal gammopathy based on serum protein electrophoresis and immunofixation, and polyclonal or restricted immunoglobulin gene rearrangements based on PCR for antigen receptor rearrangements (PARR). The findings support a nonneoplastic disorder, perhaps congenital, that may undergo malignant transformation in some dogs. Rarely, the same constellation of findings has been recognized in other breeds.

HYPOPROTEINEMIA (DECREASED [TP] IN SERUM OR PLASMA)

Disorders and conditions that cause hypoproteinemia are listed in Table 7.4.

I. Increased protein loss from the vascular space
   A. Blood loss (primarily external hemorrhage, acute or chronic)
      1. Pathogenesis: Hypoproteinemia occurs when the remaining plasma proteins are diluted by movement of extracellular fluid from extravascular space to intravascular space (Figure 3.18). This may be promoted by increased thirst or fluid replacement therapy. Hypoproteinemia will persist as long as the rate of protein loss exceeds the rate of protein production, which may be impaired by a protein-depleted state.
      2. External blood loss comprises blood donation and any external hemorrhage. External hemorrhage includes bleeding into the alimentary or urogenital tract lumens because that leads to loss of blood components from the body. This hemorrhage may be occult.
      3. With internal hemorrhage into soft tissue or body cavities, protein can return to blood via lymphatics, thus limiting hypoproteinemia, but hypoproteinemia may still develop with acute internal hemorrhage.[131]
      4. Major laboratory findings

Table 7.4. Disorders and conditions that cause hypoproteinemia
Increased protein loss from the vascular space
Blood loss*
PLN:* congenital or acquired (immune complex-mediated glomerulonephritis, amyloidosis, focal segmental glomerulosclerosis)
PLE:* small intestinal mucosal disease, lymphangiectasia, intestinal blood loss
Protein-losing dermatopathy: burns, generalized exudative skin disease including wounds
Plasma loss: peritonitis, pleuritis, vasculitis, or sepsis
Decreased protein synthesis and/or increased protein catabolism
Hepatic insufficiency*
Malabsorption or maldigestion:* intestinal mucosal disease, juvenile exocrine pancreatic insufficiency
Cachectic states:* chronic diseases, neoplasms, malnutrition, starvation
Lymphoid hypoplasia or aplasia
Inadequate transfer of passive immunity (ITPI) (also called failure of passive transfer)
Hemodilution
Excess administration of intravenous fluid
Edematous disorders: congestive heart failure, cirrhosis, nephrotic syndrome
Syndrome of inappropriate ADH secretion
Other or unknown mechanisms
Hypoadrenocorticism

* A relatively common disorder, condition, or mechanism
Note: Total protein concentrations in healthy pups, kittens, calves, and foals may be approximately 1.0–2.0 g/dL less than those found in mature animals.

a. *Panhypoproteinemia*, a nonselective hypoproteinemia with decreased concentrations of albumin and globulins, is characteristic if there are no concurrent disorders.
b. Anemia may be mild to marked and nonregenerative to regenerative, depending on the duration and magnitude of blood loss (see Chapter 3).
c. With marked, acute blood loss, there may be hypovolemia and associated prerenal azotemia; intestinal digestion of blood proteins can contribute to increased [UN] when there is substantial gastric or upper intestinal hemorrhage (see Chapter 8).

B. PLN
1. Pathogenesis: Renal glomerular damage (e.g., by immune complex or amyloid deposition) causes either a retraction of podocytes or loss of the selective permeability of the glomerular basement membrane. Either or both lesions allow larger and more negatively charged proteins through the glomerular filtration barrier, mostly albumin but also molecules as large as IgG and even IgM.[132] When proteins enter the filtrate at a rate greater than proximal tubules can resorb them, proteinuria occurs. Concurrent tubular damage contributes to proteinuria. When the rate of protein loss exceeds protein production, hypoproteinemia occurs. Persistent hypoproteinemia itself may further promote hypoalbuminemia and hypoproteinemia because of a decreased reservoir of protein and amino acids for protein synthesis. Very large proteins (e.g., α_2-macroglobulin and β-lipoprotein) typically are not lost through glomeruli, and thus selective

hypoproteinemia occurs. Because dogs and cats normally have very little β-lipoprotein, the β_2-globulin fraction typically is not relatively increased as it is in people with PLN, but α_2 globulins are.

2. Disorders
   a. Animals with PLNs (as defined in this section) have glomerulopathies that may be hereditary or acquired.
   b. Hereditary disorders
      (1) Primary glomerulonephropathy resulting from defects in glomerular basement membrane collagen type IV is reported in several breeds, including Samoyeds, Dalmatians, bull terriers, and English cocker spaniels.[133]
      (2) A familial form of PLN was reported in eight closely related, young Abyssinians. Clinical pathologic features included proteinuria, renal hematuria, nephrotic syndrome, and histologic evidence of focal proliferative glomerulonephritis.[134]
      (3) Canine familial renal amyloidosis is recognized most often in shar peis, but also has been reported in English foxhounds and beagles,[133] as well as bracchi Italiani (Italian pointers).[135] Familial amyloidosis also occurs in Abyssinian and Siamese cats.
      (4) Soft-coated wheaten terriers have an increased incidence of PLN.[136] A hereditary explanation is supported by the identification of mutations in podocyte proteins.[137]
      (5) Miniature schnauzers appear to have a predisposition for PLN that is associated with focal segmental glomerulosclerosis, a reflection of an underlying podocyte disorder.[138]
      (6) Several other breeds appear to be predisposed to disorders causing glomerular proteinuria.[133]
   c. Acquired disorders
      (1) Acquired PLN is more common than hereditary PLN. It is common in dogs but uncommon in cats.
      (2) Most acquired glomerular disease can be classified by routine and special histologic evaluation into disorders with and without immune complex deposition: (i) immune-complex–mediated glomerulonephritis that is membranoproliferative (most common in dogs) or membranous (most common in cats), and (ii) non–immune-complex glomerulopathies that include amyloidosis and focal segmental glomerulosclerosis.[133,139]
      (3) Acquired PLN is most often idiopathic, but dogs, including some shar peis, often have previous or concurrent inflammatory, infectious (viral, bacterial, protozoal, parasitic), or neoplastic disorders that may result in glomerular amyloidosis or that might produce immune complexes that deposit in glomeruli.[133,140] Sulfa drugs have been implicated in antigen–antibody complex glomerulonephritis in Doberman pinschers.[141] Because glomeruli are composed of capillaries, PLN may be caused by vasculitis, and direct renal involvement by infections or neoplasms may disrupt glomeruli and cause proteinuria.[133]
      (4) PLN in cattle is typically due to amyloid A amyloidosis and is a result of high concentrations of SAA secondary to chronic infections, especially those of bacterial origin.[142] There are rare reports of PLN and nephrotic syndrome in cattle with nonamyloid glomerulopathy and electron microscopic evidence to suggest immune-complex deposition.[143] Immune-complex glomerulonephritis in a group of 1–1.5-mo-old Japanese black calves was suspected to have been

caused by complexing of colostral- and vaccine-acquired antibodies with a *Salmonella* antigen.[144]

(5) Rare cases of immune-complex–mediated glomerulonephritis have been reported in horses.[145]

3. Major laboratory findings
   a. Mild to marked hypoproteinemia with hypoalbuminemia and normoglobulinemia is typical, but hypoglobulinemia may occur, and dogs with PLN due to chronic infections such as leishmaniasis may have hypoalbuminemia and hyperglobulinemia resulting in a [TP] that is WRI or even increased.[146]
   b. SPE results: There is usually a selective hypoproteinemia pattern with hypoalbuminemia and α_2 globulins WRI or only mildly decreased (may appear increased relative to albumin) (Figure 7.4K and L). Other protein fractions may also be decreased. Changes in the γ-globulin fraction depend on any associated inflammation and the porosity of the glomeruli.
   c. Moderate to marked proteinuria is dominated by albuminuria but includes proteins of greater molecular mass.[132,147]
   d. Evidence of renal insufficiency (azotemia or isosthenuria) may be present if the disease has destroyed enough nephrons.
   e. PLN may lead to nephrotic syndrome with hypercholesterolemia and a peritoneal transudate or edema. It may also result in a prothrombotic state associated with decreased [antithrombin], increased [antiplasmin], increased [protein C], hyperfibrinogenemia, and a prothrombotic TEG tracing.[148,149]

C. PLE
   1. Pathogenesis
      a. Protein may be lost into the intestinal tract because of increased vascular permeability caused by ulcerated or nonulcerated mucosal disease, impaired protein transport from the intestine caused by lymphatic disease, or decreased digestion or absorption of proteins in normal intestinal secretions. When the rate of protein loss exceeds the rate of production by the liver and lymphocytes, hypoproteinemia occurs.
      b. Increased protein catabolism may contribute to hypoproteinemia, as has been suggested to be important for llamas and alpacas,[76] and decreased protein intake may impair protein synthesis (see Malabsorption and Maldigestion, II.B and II.C of the next section).
      c. Intestinal blood loss (parasitic or nonparasitic) is another form of PLE.
      d. An inflammatory response (increased synthesis of PAPPs) may mask loss of globulins.
   2. Disorders
      a. Generalized small intestinal mucosal diseases: lymphoma, histoplasmosis, coccidiosis (especially *Eimeria macusaniensis* in llamas and alpacas), cryptosporidiosis, chronic inflammatory enteropathies, and acute enteritis in horses
      b. Proliferative enteritis: paratuberculosis (Johne's disease) in ruminants and camelids, and proliferative enteropathy in foals (less often adults) caused by the intracellular bacterium *Lawsonia intracellularis*;[150] in addition to protein loss, malabsorption and decreased intake may contribute to hypoproteinemia[151]
      c. Lymphatic disease: lymphangiectasia
      d. Intestinal blood loss: ulcerative enteritis, hookworms, whipworms, or neoplasms
   3. Major laboratory findings
      a. Mild to marked hypoproteinemia with hypoalbuminemia and hypoglobulinemia or normoglobulinemia

b. SPE results: The pattern may appear nonselective or selective with a disproportionately large loss of smaller proteins such as albumin (often nonselective when the animal has lost considerable body weight).
c. Other findings may indicate or suggest the inciting pathologic state (e.g., *Histoplasma* organisms, neoplastic lymphocytes in biopsy samples, fecal oocytes, or melena).

D. Protein-losing dermatopathy
1. Pathogenesis
a. Thermal or chemical burns allow plasma proteins to exude from denuded skin at a rate greater than the rate of protein production, and protein loss may impair further protein synthesis. If the animal is not seen soon after the injury, the dysproteinemia will reflect a mixture of cutaneous protein loss and an acute-phase inflammatory response.[152]
b. Trauma, including severe cutaneous abrasions and bite wounds, may denude the skin and expose large surface areas of subcutaneous tissue through which plasma proteins exude. The dysproteinemia may be influenced by associated inflammation.
c. Generalized exudative skin disease can cause a hypoproteinemic state, but the dysproteinemia probably reflects a mixture of protein loss and an inflammatory dysproteinemia.
2. Major laboratory findings
a. Early: relatively nonselective hypoproteinemia, but preferential loss of smaller proteins may decrease [albumin] more than [globulins]
b. Later: relatively nonselective hypoproteinemia masked by either an acute or chronic inflammatory dysproteinemia

E. Plasma loss caused by peritonitis, pleuritis, vasculitis, or sepsis
1. Pathogenesis
a. Pleuritis and peritonitis: Inflammation of pleura or peritoneum allows for extravasation of protein-rich fluid into the pleural and peritoneal cavities, respectively. Such protein loss is sometimes referred to as *third-space loss*. When this occurs acutely, subsequent hemodilution may cause hypoproteinemia.
b. Vasculitis: A similar loss of protein-rich fluid can occur with vasculitis; the fluid enters the interstitial fluid adjacent to the inflamed vessels.
c. Sepsis: Cytokines and endotoxins mediate vascular endothelial changes that promote increased permeability and extravasation of protein, particularly albumin.[153,154]
d. Smaller proteins may be lost disproportionately to large molecules.
e. A NAPP response may contribute to the hypoproteinemia.
2. Major laboratory findings
a. Early: hypoalbuminemia and hypoglobulinemia or normoglobulinemia
b. Later: similar, but hypoproteinemia may be masked by an acute or chronic inflammatory dysproteinemia

II. Decreased protein synthesis and/or increased protein catabolism (Figures 7.4J and 7.5L)
A. Hepatic insufficiency (hepatic failure)
1. Pathogenesis: A marked reduction in functional hepatic mass (< 20 % remaining) decreases the synthesis of nearly all plasma proteins except immunoglobulins. Normal protein catabolism combined with decreased protein synthesis produces the hypoproteinemia.
2. Disorders
a. Cirrhosis (fibrosis)

b. Hepatic necrosis, degeneration, or inflammation (not acute)
c. Hepatic atrophy secondary to portosystemic shunts
d. Neoplasms that extensively damage the liver

3. Major laboratory findings
a. Hypoproteinemia, hypoalbuminemia, and normo-, hypo-, or hyperglobulinemia
b. SPE results: The pattern may be nonselective or selective with a relative excess or increase in β_2 globulins, γ globulins, or both. This may cause *beta–gamma bridging*, a diagnostically nonspecific finding characterized by loss of a distinct separation between β_2- and γ-globulin fractions.[155] Most theories for this pattern involve increased immunoglobulin production with increased amounts of IgM and IgA migrating in the β_2-globulin fraction and blending with increased IgG in the γ-globulin fraction. Because the liver removes antigenic material and IgA from the portal blood, hepatic insufficiency may allow antigenic material to bypass hepatic clearance and induce a systemic immune response (increased IgM, IgA, or IgG). Immunoglobulin production may alternatively increase as part of an inflammatory hepatopathy, or immunoglobulin clearance and degradation may be decreased.[156]

4. Other laboratory findings of hepatic dysfunction or disease include increased bile acid or ammonium concentrations, decreased urea ([UN]) or cholesterol concentrations, increased serum or plasma activities of liver-associated enzymes, hyperbilirubinemia, and prolonged coagulation times.

B. Malabsorption or maldigestion
1. Terms
a. *Maldigestion* is impaired breakdown of lipids, proteins, and carbohydrates. It may be caused by decreased exocrine pancreatic secretions or gastrointestinal disorders that reduce enzymatic activity in the lumen or intestinal brush border.
b. *Malabsorption* is impaired uptake of nutrients that are in the intestine. This is usually caused by acquired abnormalities of the intestinal wall.
c. Because maldigestion promotes malabsorption and malabsorption can alter digestion, these conditions are difficult to separate. The term *malabsorption* is often used to include both processes.
d. When hypoproteinemia is associated with intestinal disease, the disorder is often considered a PLE whether or not factors other than protein loss contribute significantly to the hypoproteinemia.

2. Pathogenesis
a. Maldigestion and malabsorption reduce intestinal uptake of basic body fuels to replace fuels used by metabolic pathways for daily energy.
b. Once depleted, protein catabolism and the use of amino acids for gluconeogenesis lead to a deficiency in energy and amino acids for protein synthesis in hepatocytes, lymphocytes, and other cells. When catabolism exceeds production, hypoproteinemia occurs.
c. Concurrent protein loss may occur because of intestinal mucosal damage (see PLE section above).

3. Disorders
a. Small intestinal diseases with generalized mucosal involvement (see PLE disorders)
b. Exocrine pancreatic insufficiency: Juvenile-onset exocrine pancreatic insufficiency has been associated with hypoproteinemia and hypoalbuminemia in several breeds of dogs,[157,158] but these abnormalities do not appear typical of exocrine pancreatic insufficiency in adults.

4. Major laboratory findings
   a. Hypoproteinemia with hypoalbuminemia and normoglobulinemia or hypoglobulinemia
   b. SPE results: hypoalbuminemia; globulin findings depend on intestinal protein losses and concurrent inflammation
   c. Other findings specifically related to malabsorption or maldigestion (see Chapter 15)
      (1) Low [folate] may occur with malabsorptive states of the proximal small intestine, hypocobalaminemia with malabsorptive states of the distal small intestinal, or both with diffuse intestinal disease.
      (2) With exocrine pancreatic insufficiency, trypsin-like immunoreactivity is decreased and hypocobalaminemia is common.

C. Cachectic states
   1. Pathogenesis: When the rate of protein catabolism exceeds protein production, the negative protein status causes hypoproteinemia. However, glucose and most serum protein concentrations (especially albumin) tend to be maintained at the expense of other tissues, so this hypoproteinemia is expected only with severe weight loss or emaciation, and even then it may not be present. Underlying or concurrent conditions may contribute to hypoproteinemia through multiple mechanisms, and dehydration may mask it.
   2. Disorders
      a. Chronic diseases such as chronic infections and malignant neoplasms; protein catabolism for gluconeogenesis may be a significant contributor to hypoproteinemia in ill llamas and alpacas[76]
      b. Marked malnutrition or starvation[159]
   3. Major laboratory findings
      a. Hypoproteinemia and hypoalbuminemia with normoglobulinemia, hypoglobulinemia, or hyperglobulinemia
      b. SPE results: The pattern typically appears selective with hypoalbuminemia, but globulin findings vary.
      c. Other findings are dependent on the primary pathologic state but may include anemia and increased [UN].

D. Lymphoid hypoplasia or aplasia
   1. Pathogenesis: In health, B lymphocytes and plasma cells produce immunoglobulins but not other major plasma proteins. A mild hypoproteinemia might be created by lymphoid hypoplasia if concentrations of other proteins are WRI.
   2. Expected dysproteinemia
      a. The [TP] is WRI to slightly decreased. The [albumin] is WRI, and the [globulins] is WRI or slightly decreased.
      b. SPE results: decreased [γ globulins] from decreased IgG production
   3. Disorders
      a. Severe combined immunodeficiency in horses (Arabian, Appaloosa, Caspian) and dogs (basset hounds, Cardigan Welsh corgis, Jack Russell terriers, and Frisian water dogs), and common variable immunodeficiency in horses (see also Lymphopenia in Chapter 2); quantitative immunoglobulin assays are needed to confirm decreases of immunoglobulins (see the Immunoglobulin section)
      b. Chemotherapy or infections may cause lymphoid hypoplasia.[160]

E. Miscellaneous: A minority of cats with idiopathic hepatic lipidosis but not overt hepatic dysfunction have mild hypoalbuminemia at presentation, and more develop it soon after

the initiation of therapy.[161,162] It has been suggested that cats, as obligate carnivores, develop impaired protein synthesis when anorexic,[161,163] but data regarding effects of anorexia or protein restriction on serum [albumin] in cats were not found. Hepatic dysfunction, inflammation, and diagnostic and therapeutic interventions may contribute to mild hypoalbuminemia in these cats.

III. Inadequate transfer of passive immunity (ITPI) (see Immunoglobulins section for additional information): Neonates who fail to absorb colostral proteins have lower serum or plasma [TP], primarily because of lower [IgG]. However, dehydration or production of PAPPs during inflammation can increase the [TP] and mask ITPI.

IV. Hemodilution: increased ECF volume
   A. By itself, increased ECF is a very uncommon cause of hypoproteinemia, but it may lower protein concentrations that were already decreased because of another problem.
   B. Disorders or conditions
      1. Excess administration of intravenous fluid (too fast or too much)
      2. Edematous disorders (congestive heart failure, cirrhosis, and nephrotic syndrome)
      3. Syndrome of inappropriate antidiuretic hormone secretion
      4. Use of plasma expanders, when extravascular fluid is pulled into plasma soon after administration (does not increase ECF volume but dilutes the plasma protein concentration)
      5. Diabetes mellitus, when marked persistent hyperglycemia draws extravascular H_2O into the blood by osmosis, thus diluting solutes including proteins

V. Other or unknown mechanism
   A. Mild to severe hypoproteinemia due to hypoalbuminemia of unknown cause occurs in dogs with hypoadrenocorticism,[164] and it may be more severe in those dogs with glucocorticoid but not mineralocorticoid deficiency.[165] Nonselective hypoproteinemia and findings suggestive of protein-losing enteropathy have also been reported.[164] Intestinal protein loss is often cited as a contributing cause based on frequent reports of diarrhea, with or without blood, coupled with reported positive effects of glucocorticoids on enteric mucosal barrier function.
   B. Serum concentrations of total protein, albumin, and globulins in Alaskan sled dogs were lower 20–60 min after an endurance race than before it, but RIs appropriate for the laboratory and for resting Alaskan sled dogs, a genetically distinct population,[166] were not available to determine if values actually became low.

HYPERALBUMINEMIA

Disorders and conditions that cause hyperalbuminemia are listed in Table 7.5.

I. Hemoconcentration from dehydration: decreased ECF volume
   A. Decreased plasma H_2O leads to greater concentrations of those substances (including albumin and erythrocytes) that have circulating life spans longer than the time it took to become dehydrated.
   B. Hemoconcentration is the most common reason for hyperalbuminemia. Concurrent hyperproteinemia and perhaps erythrocytosis are expected.

Table 7.5. Disorders and conditions that cause hyperalbuminemia
Hemoconcentration*
Increased albumin synthesis induced by glucocorticoid drugs or hormones
Paraneoplastic hyperalbuminemia (hepatocellular carcinoma)

* A relatively common disorder, condition, or mechanism
Note: If [albumin] is determined by the BCG method, the BCG dye may bind to proteins other than albumin and thus yield pseudo-hyperalbuminemia.

C. Hemoconcentration can occur without dehydration when there is a shift of intravascular fluid to the extravascular compartment (e.g., endotoxemia). If extravasation of water is greater than that of albumin, [albumin] increases. However, increased vascular permeability to albumin may prevent hyperalbuminemia and even cause hypoalbuminemia despite an erythrocytosis of hemoconcentration.

II. Induced synthesis by glucocorticoid therapy
   A. In dogs given prednisone (0.55 mg/kg, q12h) for 4 wk, the [albumin] and [TP] increased more in the prednisone-treated dogs than in a control group.[167] The [albumin] might have increased because of increased production[168] or, possibly, an increased albumin life span.
   B. One study reported a large increase in [albumin] (~2 g/dL) after 4–5 d of treatment with methylprednisolone (4 mg/kg IM q24h), but the [albumin] was measured by a biuret method after Na_2SO_3 precipitation, so the increase may have included proteins other than albumin (e.g., Hp).[169]

III. Paraneoplastic hyperalbuminemia: Rarely, hyperalbuminemia may occur because of increased albumin production by a hepatocellular carcinoma.[170]

IV. Falsely increased [albumin] determined by the BCG dye method
   A. BCG dye preferentially binds to albumin. However, it also binds to some α globulins and β globulins, and thus a measured [albumin] represents the dye-binding to albumin and some globulins.[171] The binding of BCG to globulins is reduced if the assay's incubation time is reduced.[172] Falsely increased [albumin] is also found in heparinized plasma because of the binding of BCG to Fbg.[44]
   B. This error is typically a small percentage of the [albumin], and therefore clinically insignificant. However, the relative error is greater with marked hypoalbuminemia, in which case the magnitude of hypoalbuminemia may be underestimated and the [albumin] from SPE may be considerably less than the BCG value.

HYPOALBUMINEMIA

I. Hypoalbuminemia commonly occurs with hyperproteinemia and hypoproteinemia but also can be seen when there is normoproteinemia.

II. The disorders and conditions that cause hypoalbuminemia are listed in Table 7.6. See the relevant Hyperproteinemia and Hypoproteinemia sections for further discussion of the pathogeneses of hypoalbuminemic states.
   A. As described in the Hypoproteinemia section, hypoalbuminemia commonly occurs with hypoproteinemia and is usually caused by decreased production of albumin, increased loss of plasma albumin, or a combination of these mechanisms.

Table 7.6. Disorders and conditions that cause hypoalbuminemia
Decreased albumin synthesis
Inflammation*
Hepatic insufficiency*
Malabsorption and maldigestion*
Cachectic states*
Hypergammaglobulinemia
Increased albumin loss
Blood loss*
PLN:* congenital or acquired (immune complex-mediated glomerulonephritis, amyloidosis, focal segmental glomerulosclerosis)
PLE:* small intestinal mucosal disease, lymphangiectasia, intestinal blood loss
Protein-losing dermatopathy: burns, generalized exudative skin disease
Plasma loss from vessels: peritonitis, pleuritis, vasculitis, or sepsis
Hemodilution
Excess administration of intravenous fluid
Edematous disorders: congestive heart failure, cirrhosis, nephrotic syndrome
Syndrome of inappropriate ADH secretion
Other or unknown mechanisms
Hypoadrenocorticism

* A relatively common disorder, condition, or mechanism
Note: [Albumin] in healthy pups, kittens, calves, and foals may be approximately 0.5–1.5 g/dL less than those found in mature animals.

B. Hypoalbuminemia may also occur in hyperproteinemic states not caused by hemoconcentration, or in similar normoproteinemic states.
   1. Inflammatory hypoalbuminemia
      a. Inflammation causes hypoalbuminemia partly because albumin is a NAPP and its production decreases during inflammatory states.[173] The effect on serum or plasma [albumin] varies with the species because of differences in albumin's plasma half-life, which is generally longer in species of larger animals.
         (1) The plasma half-life of albumin in healthy dogs is about 5–12 d, being on the longer end of the range with low protein intake.[1,2] In healthy horses, the half-life is estimated to be about 19 d.[4]
         (2) Hypoalbuminemia from a reduction of albumin production alone is therefore not expected until inflammation has persisted for a few days in dogs or for 1–2 wk in horses. It will be mild at first, potentially progressing to a moderate degree. Marked hypoalbuminemia or abrupt development of hypoalbuminemia suggests other mechanisms.
         (3) In dogs, experimentally induced septic arthritis or sterile muscular abscesses led progressively to 25–50 % decreases in [albumin] in 2–4 d (estimated from published graph);[174] in another study, similar decreases occurred and reached a nadir 5 d after induction of a sterile abscess.[78]

b. Hypoalbuminemia commonly occurs in clinical cases of sepsis (particularly Gram negative) and systemic inflammatory response syndrome (SIRS) with or without changes in [TP].[175–177] Mechanisms of the hypoalbuminemia in these inflammatory conditions are multifactorial and depend on the clinical manifestations of disease, but likely include cytokine- and LPS-mediated increases in vascular permeability (paracellular and transcellular) in addition to LPS-mediated suppression of albumin synthesis.[178,179] With sepsis, the transcapillary escape rate of albumin may increase to > 3 times that in health.[154]

2. The pathogenesis of hypoalbuminemia concurrent with lymphoid neoplasms that create paraproteinemia varies and may relate to inflammatory cytokines, response to increased COP caused by hyperglobulinemia, or damaged tissues such as liver, intestine, or kidneys.

HYPERGLOBULINEMIA (see the Hyperproteinemia section and Table 7.3)

I. Hyperglobulinemia is typically present concurrently with hyperproteinemia, and the reason for the hyperproteinemia is typically the hyperglobulinemia. However, it is not uncommon to find hyperglobulinemia with normoproteinemia because of concurrent hypoalbuminemia.

II. There are four major concepts to consider:
   A. The major causes of hyperglobulinemia are hemoconcentration, inflammation (especially chronic), and lymphoid neoplasms that create paraproteinemia.
   B. By way of routine clinical chemistry methods, the [globulins] is determined by subtraction: [TP] – [albumin]. Thus, errors in those values can result in erroneous [globulins].
   C. Because the routine [globulins] represents the sum of the concentrations of all proteins other than albumin, hyperglobulinemia may result from the increased concentration of one or more different globulins.
   D. SPE results typically differentiate the hyperproteinemia of hemoconcentration from hyperglobulinemias of inflammation and lymphoid neoplasms, but not all inflammatory hyperglobulinemias can be differentiated from neoplastic lymphoid hyperglobulinemias.

HYPOGLOBULINEMIA (see the Hypoproteinemia section and Table 7.4)

I. For animals other than neonates, hypoglobulinemia commonly occurs concurrently with hypoproteinemia and might be seen with normoproteinemia, but it is not expected with hyperproteinemia if RIs are appropriate.

II. As with the hypoproteinemic disorders, the major causes of hypoglobulinemia fall into two categories:
   A. Production of globulins is decreased because of decreased immunoglobulin synthesis by plasma cells (congenital or acquired) or decreased production of many different globulins by hepatocytes. Albumin production may be decreased in either case.
   B. Loss of plasma globulins is increased because of blood loss or loss via intestinal mucosa, skin, or sometimes glomeruli. Albumin loss would occur concurrently, and often to a greater degree with causes other than hemorrhage.

III. See Physiologic processes and Table 7.3 and 7.4 legends for age- and breed-related variations in the [globulins].

ALBUMIN:GLOBULINS (A:G) RATIO

I. Some laboratories report an A:G ratio that is unitless and calculated by dividing [albumin] by [globulins] (using the same units for each). If [albumin] and [globulins] were equal, the A:G ratio would be 1.

II. Increases and decreases in the A:G ratio indicate the presence of a dysproteinemia, but [albumin] and [globulins] each must be assessed to interpret the abnormality (see previous sections).
   A. An increased ratio may reflect hyperalbuminemia, hypoglobulinemia, both, or trends toward both.
   B. A decreased ratio may reflect hypoalbuminemia, hyperglobulinemia, both, or trends toward both, but it might also occur with hypoglobulinemia if the hypoalbuminemia is more severe. A low A:G ratio is expected in cats with feline infectious peritonitis, but it is not specific for the condition; a ratio > 0.8 may help exclude FIP.[180]
   C. The A:G ratio might be an index of health in dairy cows, lower A:G ratios prior to drying off being associated with subsequent lower milk yields, decreased fertility, and greater milk somatic cell counts.[181]
   D. Abnormal [albumin] and/or [globulins] may exist when this ratio is WRI. For example, both values may be either increased (e.g., dehydration) or decreased (e.g., PLE, hemorrhage) to similar degrees. Reference values for recognizing an increased or decreased A:G ratio are dependent on the assays used for [albumin] and [TP], so reference values are likely to vary among laboratories.
   E. The A:G ratio in healthy greyhounds and whippets is expected to be lower than in most canine breeds because of lower [globulins] than most dogs.[17]

POSITIVE ACUTE-PHASE PROTEINS (PAPPs)

I. General concepts
   A. Major aspects of the stimulated production of PAPPs are described in earlier sections of this chapter (see General Concepts for Total Protein, Albumin, and Globulins, Section II.C). Physiologic aspects of PAPPs and clinical application of PAPP assays in domestic mammals have been reviewed.[21–23,182–186]
   B. Essentially any injury that produces an acute inflammatory reaction can increase concentrations of the PAPPs as part of the innate immune response. These injuries can be due to infections (e.g., bacterial, viral, fungal, or protozoal) or noninfectious conditions (e.g., physical trauma, burns, or necrosis). As a group of proteins, the increased concentration of PAPPs can increase [TP] and [globulins]. Individually, the magnitude of increase varies considerably.
   C. Value of PAPP results:
      1. Other methods of detecting inflammation (i.e., pyrexia or neutrophilia) may be too insensitive or nonspecific for the pathologic state of interest.
      2. They provide another method of monitoring an inflammatory process, either for therapeutic decisions or for prognostic classification.
      3. Some can be obtained with point-of-care testing, allowing for rapid patient-side decision-making.

D. Other factors to consider when interpreting concentrations of PAPPs:
   1. Because chronic inflammatory disease is the persistence of acute inflammatory disease, concentrations of PAPPs may remain increased in animals with chronic inflammatory disorders.
   2. A panel of PAPPs is more informative than a single protein. Including a major, moderate, and NAPPs in a diagnostic panel can enhance diagnostic information, as can combining information from PAPPs with shorter and longer response times (e.g., SAA and fibrinogen in horses) and interpreting along with other inflammatory markers such as [neutrophil], toxic change, and serum [Fe].[184]
   3. As exemplified by lower αAG concentrations in dogs < 2–3 mo of age and mildly increased [SAA] in newborn foals compared to adults,[184] interpretation of these PAPPs requires caution when patients are young.[187] Breed differences may also occur, as suggested by lower [Hp] in greyhounds compared to nongreyhounds (unmatched for sex)[20] and mildly greater values in miniature Schnauzers compared to other breeds of dog.[188]
   4. Concurrent with pathologic states that increase concentrations of PAPPs, there may be processes that decrease their concentrations.
      a. Disorders that cause the loss of plasma proteins (e.g., blood loss or glomerular disease) can lower PAPP concentrations.
      b. Disorders that activate plasmin or thrombin can lower [Fbg] by fibrinogenolysis or coagulation, respectively.
      c. Intravascular hemolysis lowers [Hp], though much less in the dog compared to people.[189]

II. Fibrinogen
   A. Physiologic processes
      1. Fbg is a plasma protein that is produced by hepatocytes.
      2. When enzymatic processes in plasma convert prothrombin to thrombin, thrombin then promotes the conversion of soluble Fbg to insoluble fibrin. In vivo, interactions of fibrin, platelets, and endothelial cells help prevent blood loss from blood vessels.
      3. Because Fbg is a PAPP, plasma [Fbg] is expected to increase during inflammatory states. However, when inflammation is concurrent with coagulation and fibrinolysis, increased Fbg consumption may mask increased Fbg production and vice versa.
   B. Analytical concepts
      1. Heat-precipitant methods
         a. Principle: Blood can be heated to selectively precipitate Fbg from plasma, thus allowing either (i) estimation of [Fbg] by measuring the difference in the [TP_{ref}] in a sample before and after precipitation or (ii) calculation of the percentage of plasma volume filled by the precipitate (the "Fbg-crit").
         b. Clinical use: These methods are used mostly as screening tests for plasma hyperfibrinogenemia. Although Fbg is a minor or moderate PAPP in all domestic species, this test is usually applied only to ruminant and equine samples because inflammation is less reliably detected by inflammatory leukograms in these species compared to dogs and cats. The "Fbg-crit" method may also detect hypofibrinogenemia.
         c. Refractometric method (Schalm's method)
            (1) As originally described, two microhematocrit tubes are filled at least three-quarters full with EDTA-anticoagulated blood. The tubes are spun in a microhematocrit centrifuge (as for hematocrit) for 5 min.

(a) The [pTP_{ref}] for one tube is measured to the nearest 0.1 g/dL.
(b) The other tube is placed in a 56–58 °C H_2O bath for 3 min and then spun in a microhematocrit centrifuge (for at least 1 min) to pack the precipitated Fbg. The [TP] of the Fbg-depleted plasma is then measured via refractometer (to the nearest 0.1 g/dL). Note: Because precipitated Fbg is no longer in solution, it does not alter the refractive index; therefore, centrifugation of the heated tube is not essential.
(c) The estimated [Fbg] = [pTP_{ref}] of the first tube – [TP_{ref}] of the second tube. Example: 7.0 g/dL – 6.7 g/dL = 0.3 g/dL.

(2) A modification is to initially spin only one of the microhematocrit tubes and to heat the second (as described) without centrifugation. After heating, centrifugation is used to pack the cells and precipitated Fbg prior to refractometry of the Fbg-depleted plasma. Data to support this modification were not found.
(3) Unit conversion: mg/dL × 0.01 = g/L (SI unit);[27] reporting heat-precipitant Fbg results in mg/dL units (e.g., 300 mg/dL instead of 0.3 g/dL), as has been commonly done, implies much greater precision than the assay can deliver.
(4) The method's analytical sensitivity (i.e., capability of detecting small changes) is inadequate to document hypofibrinogenemia. As each refractometric reading is at best only accurate to the nearest 0.1 g/dL, the calculated [Fbg] should be considered to be at best within 0.2 g/dL of the true value. In most species, the lower reference limit is 0.1–0.3 g/dL.

d. Packed Fbg layer measurement (Millar's method)[190]
(1) Two microhematocrit tubes of blood are spun as for measuring hematocrit, then heated in a 56–58 °C H_2O bath for 3 min and spun again.
(2) Using a microscope with an ocular reticle, the length of the packed precipitate layer is measured to the nearest 0.1 mm, as is the length of the original plasma layer (including the precipitate). The "Fbg-crit" = length of the precipitant layer / length of the plasma layer × 100. The mean of the results for the two tubes is reported. Measurement should promptly follow centrifugation to maintain a crisp Fbg-fluid interface.
(3) Units: mL/100 mL or %
(4) The IDEXX VetAutoread Hematology Analyzer generates an automated measurement of a tube's packed Fbg layer and somehow converts it to a value for [Fbg] in units of mg/dL. This method had a positive proportional bias compared to Millar's method when [Fbg] was > 0.5 g/dL.[191] Values as low as 80 mg/dL (hypofibrinogenemia for most species) are reported (instrument operator's guide), and the measurement precision allows detection of hypofibrinogenemia in some species.[191]

2. Thrombin time, von Clauss modification
a. This method is typically used with citrated plasma samples to detect hypofibrinogenemia as part of a hemostasis profile (see Chapter 5).
b. Principle: A [Fbg] can be determined from the time required for fibrin formation after the addition of a high concentration of thrombin to diluted citrated plasma. This time primarily depends on the [Fbg]; the lower the [Fbg] is, the longer will be the time until clot formation.
3. Other automatable turbidometric methods

a. Ammonium sulfate precipitation: [Fbg] is proportional to the change in absorbance before and after precipitation of Fbg from plasma by an ammonium sulfate reagent. Only small amounts of other proteins precipitated from the plasma of healthy dogs.[192] The assay appeared capable of detecting hypofibrinogenemia.
b. Vancomycin precipitation: [Fbg] is proportional to the change in absorbance before and after vancomycin-induced precipitation of Fbg from EDTA or citrate-anticoagulated plasma. Preliminary assessment in dogs showed that heparin anticoagulation yielded no detectable [Fbg].[193]

4. People have attempted to calculate a [Fbg] by determining the difference between the [pTP_{ref}] of EDTA-anticoagulated plasma and the [sTP_{ref}] from a serum sample. Such a method is not recommended because solutes other than Fbg (including the anticoagulant solution) alter the refractive index and thus cause differences between plasma and serum [TP] (see the section on Analytical Principles for Total Protein, Albumin, and Globulins). Preanalytical errors can arise when two different samples are collected and compared.

C. Hyperfibrinogenemia (plasma) (Table 7.7)
1. Two major causes:
a. Inflammation: increased Fbg production by the liver
b. Hemoconcentration (decreased plasma H_2O volume)
2. The [TP] is also usually increased with these disorders but may be WRI. Unless Fbg consumption is increased enough, the [Fbg] increases relatively more than the [TP] during inflammation. With dehydration, the increases in the [TP] and [Fbg] should be relatively the same, e.g., they both increase by 5 %. These concepts led to the development of the PP:F ratio (Eq. 7.1a).[194] The ratio was simplified a few years later by not subtracting the [Fbg] from the [TP_{ref}] (Eq. 7.1b).[195] In theory, subtracting [Fbg] from the [TP_{ref}] should be a better ratio as it is comparing increased concentrations of proteins other than Fbg to the increased concentration of Fbg.

$$\text{PP:F ratio} = \frac{[\text{plasma TP}] - [\text{fibrinogen}]}{[\text{fibrinogen}]} \quad (\mathbf{7.1a})$$

$$(\text{TP:Fib})_p \text{ ratio} = \frac{[\text{plasma TP}]}{[\text{fibrinogen}]} \quad (\mathbf{7.1b})$$

Example: [pTP] = 8.8 g/dL and [fibrinogen] = 0.8 g/dL

$$\text{PP:F ratio} = \frac{8.8 \text{ g/dL} - 0.8 \text{ g/dL}}{0.8 \text{ g/dL}} = \frac{8.0 \text{ g/dL}}{0.8 \text{ g/dL}} = 10$$

$$(\text{TP:Fib})_p \text{ ratio} = \frac{8.8 \text{ g/dL}}{0.8 \text{ g/dL}} = 11$$

Table 7.7. Disorders and conditions that cause hyperfibrinogenemia

Increased fibrinogen production
Inflammation*
Hemoconcentration*

* A relatively common disorder, condition, or mechanism

3. PP:F ratio or $(TP:Fib)_p$ ratio interpretation
   a. These ratios may help in differentiating hyperfibrinogenemia of inflammation or dehydration.
   b. The original interpretive guides and RIs were established with the PP:F ratio.[194,196] The PP:F ratios in healthy mammals varied among species and by age group, mostly because of different total protein concentrations.[197]
      (1) Cattle: If the ratio is > 15, hyperfibrinogenemia is probably caused by dehydration. If the ratio is < 10, hyperfibrinogenemia is probably caused by inflammation.
      (2) Horses: If the ratio is > 20, hyperfibrinogenemia is probably caused by dehydration. If the ratio is < 15, hyperfibrinogenemia is probably caused by inflammation.
      (3) If the simpler calculation, for $(TP:Fib)_p$ ratio, is used, a "1" should be added to guideline values to be consistent with guidelines determined by using the PP:F ratio (e.g., > 16 in cattle is probably dehydration).
   c. Guidelines are based on the assumptions that a healthy animal's [TP] and [Fbg] are WRI, and then one of two things happens: the animal (i) becomes dehydrated or (ii) develops an inflammatory disease.
      (1) Dehydration increases concentrations of all proteins to the same degree, and thus the PP:F and $(TP:Fib)_p$ ratios do not change.
      (2) Inflammation increases production of Fbg and some other proteins but also decreases concentrations of some proteins. The PP:F and $(TP:Fib)_p$ ratios decrease because the denominator increases relatively more than the numerator.
   d. Additional factors to be considered
      (1) Concurrent dehydration and inflammation make interpretation of the ratios more difficult. Increased Fbg consumption in the pathologic state will also cloud the issue.
      (2) Dehydration by itself causes only minor increases in [Fbg].
      (3) The aforementioned ratio guidelines are not appropriate for calves and foals because the ratio RIs are for mature animal values. They also are not appropriate for cattle and horses that have a concurrent pathologic state that causes hypoproteinemia.
      (4) The accuracy of refractometric values is at best ± 0.1 g/dL. Thus, the accuracy of the calculated ratio is probably better with a higher [Fbg]. Even then, however, ratios should be considered estimates.

D. Hypofibrinogenemia (plasma) (Table 7.8)

Table 7.8. Disorders and conditions that cause hypofibrinogenemia

Increased fibrinogen consumption
Intravascular coagulation (localized or disseminated)*
Increased fibrinogenolysis
Decreased synthesis of fibrinogen
Hepatic insufficiency
Afibrinogenemia, congenital hypofibrinogenemia (rare)

* A relatively common disorder, condition, or mechanism

1. Heat-precipitation techniques may lack sufficient analytical sensitivity and precision to be used for detection or confirmation of hypofibrinogenemia, e.g., Schalm's method. The common assay for detecting hypofibrinogenemia is the quantitative von Clauss modification of the thrombin time (see Chapter 5).
2. Causes of hypofibrinogenemia (more information in Chapter 5)
   a. Increased consumption of Fbg
      (1) Intravascular coagulation: local or disseminated
      (2) Increased fibrinogenolysis: uncommon but may occur with fibrinolytic therapy, consumptive coagulation, or certain envenomations
   b. Decreased synthesis of Fbg
      (1) Hepatic insufficiency: reduction in hepatic function must be marked before hypofibrinogenemia occurs
      (2) Inherited or congenital disorders: afibrinogenemia, hypofibrinogenemia

III. Other PAPPs
   A. Analytical concepts
      1. Like most plasma proteins, the PAPPs are relatively stable analytes when stored frozen. Serum has been the preferred sample for assessment of most PAPPs, but [Fbg] must be measured in plasma because Fbg is consumed during clotting, and plasma is preferred for measuring Cp activity because a substantial percentage of Cp leaves the fluid phase during clotting.[198] Heparinized plasma is acceptable for several assays, but anticoagulants (EDTA, heparin, and citrate) have been reported to cause a variety of effects on certain PAPP results. The effects of Hgb, bilirubin, or lipemia on the assays vary with the analyte and the assays used. Hemolyzed samples must be avoided when measuring [Hp] because the common assay is based on Hgb binding.
      2. A variety of analytical methods are used to measure increased concentrations of specific PAPPs. Some of the immunologic methods are species specific. Human assays may not work in domestic mammals; they must be validated and optimized for veterinary samples.[199] In health, the concentrations of some PAPPs are near the detection limits of the assays, so detecting decreased concentrations may not be possible.
         a. CRP is usually measured by immunologic methods, including immunoturbidimetry, ELISAs, immunofluorometry, and latex agglutination. Point-of-care assays include a magnetic two-site immunoassay, a lateral flow immunoassays, a reverse passive latex agglutination test, immunochromatographic strip assays using polyclonal or monoclonal antibodies to canine CRP, and a quantitative sandwich assay available on an in-clinic chemistry analyzer. Immunoturbidimetric methods using antibodies designed to bind human or canine CRP have been assessed and are in use for dogs.[200–202] Canine CRP assays and measurement issues have been reviewed.[203] A turbidimetric method based on the binding of CRP to phosphocholine in a fat emulsion has been tested in horses and might have cross-species applicability.[204]
         b. Hp assays commonly have been based on Hp's high affinity for Hgb. The binding is detected either via spectral differences of bound versus unbound Hgb or by diminished peroxidase activity. Because of the species differences in Hp's amino acid compositions, immunoassays designed for human Hp may or may not detect a domestic mammal's Hp. A human immunoturbidimetric assay showed promise for use in dogs.[205] Antibodies generated to Hp of various species have been

generated and used in ELISAs and more-efficient immunoturbidometric assays, the latter offering the potential for widespread automation, minimal interference from hemolysis, and a limit of detection that is low enough for quantitation of [Hp] in healthy cattle.[206]

c. SAA is measured with immunologic assays: automated immunoturbidimetric assays or ELISAs. Assays using anti-(canine SAA) or anti-(feline SAA) antibodies have been developed, but there is sufficient homology in the human, canine, feline, and equine proteins to cross-react with some polyclonal and monoclonal anti-(human SAA) antibodies used in human assays.[207–209] Similarly, there is cross reactivity between canine and feline species with anti-(canine SAA) and anti-(feline SAA) antibodies.[210] The clinical utility of a point-of-care lateral flow immunoassay for equine SAA has been described; limitations included a low upper working range, imprecision at greater [SAA], inter-batch inconsistency, and unreliable results with whole blood samples.[211]

d. αAG is measured with species-specific immunoassays, single radial immunodiffusion assays, ELISAs, and immunoturbidimetric assays.[212]

e. Cp assays are typically manual or automated spectrophotometric assays of enzymatic activity (not mass concentration) based on the oxidation of specific substrates by Cp.[213] Plasma is preferred over serum because clotting causes serum Cp activity (and copper concentration) to be substantially decreased.[198] Cp activity decreased rapidly after 3 d of storage at room temperature or 4 °C.[214]

f. Ferritin

(1) Generally, species-specific immunoassays are needed to measure serum or plasma [ferritin]. Assays have been developed specifically for canine,[215] feline,[216] equine,[217] and bovine ferritin.[218] Endogenous ferritin-binding proteins can inhibit the immunoassays for feline ferritin.[219] Similar reports were not found for other species, but ferritin-binding proteins such as α_2-macroglobulin, antiferritin autoantibody, apolipoprotein B, and fibrinogen have been reported in various species.[219]

(2) Occasionally, the cross-reactivity is sufficient to measure ferritin concentrations of species other than the one for which the assay was developed, e.g., the equine ferritin assay can be used to measure concentrations of rhinoceros, tapir, and lemur ferritin. When possible, the ferritin used in calibrating (standard) solutions should be isolated from the species of interest, e.g., use of lemur ferritin to calibrate an assay for lemur ferritin that uses an anti-(equine ferritin) antibody.

B. Increased concentrations of PAPPs other than Fbg (Table 7.9)

1. PAPPs are nonspecific indicators of inflammation, so concentrations are increased in a wide variety of disorders and conditions; however, there are also noninflammatory causes of increases. As examples, hyperferritinemia occurs with neoplasms (histiocytic sarcoma, lymphoma), hepatic disease, and inflammatory conditions including immune-mediated hemolytic anemia.[220] The magnitude of hyperferremia in these conditions overlaps enough that other means of establishing a diagnosis are required in any particular individual with hyperferritinemia.
2. Potential magnitudes of increase are provided in Table 7.2 in terms of major, moderate, or minor responders. Increased [Hp] may be sufficient to produce a recognizable increase in the serum [TP] by itself, whereas increased concentrations of the other PAPPs (other than Fbg) typically are not.

Table 7.9. Disorders and conditions that cause increased concentrations of positive acute-phase proteins (PAPPs)[a]
Inflammation:* infectious or noninfectious, localized or systemic
Neoplasms:* hematologic neoplasms, carcinomas, nonhematologic sarcomas
Treatment with certain drugs
Certain anthelmintics (see text) (haptoglobin)
Phenobarbital (αAG)
Glucocorticoids* (haptoglobin)
Hyperadrenocorticism (haptoglobin)
Physiologic states
Lactation in cattle (CRP)
Pregnancy in bitches*
Exercise (ferritin in horses)
Transportation and housing changes in cattle and dogs (SAA, Hp, CRP)
Increased iron stores: hemochromatosis, hemosiderosis, chronic hemolysis (ferritin)

* A relatively common disorder, condition, or mechanism

[a] Unless a specific PAPP is indicated, all PAPPs for a particular species may be increased (see Table 7.2 for some species differences). Not all PAPPs have been assessed in each species and condition.

3. Studies of PAPPs often yield decision thresholds that might be used to reach a conclusion about a particular disorder, but they typically are not applicable to other assays or to the same assays when different calibrators are used. In addition, because they are nonspecific, consideration must be given to the presence of concurrent conditions that may affect PAPPs independently of the condition of interest.
4. Inflammation: In addition to providing nonspecific evidence of inflammation that could be focal or systemic, increased concentrations of PAPPs can help differentiate if disease in a particular organ is likely to be inflammatory or noninflammatory, as well as help with patient or herd monitoring and prognostication. Selected examples of these uses of PAPP measurement are provided below.
    a. Characterizing disorders as inflammatory or noninflammatory
        (1) Increases in [CRP] were clearly greater in most dogs with bacterial pneumonia than in most dogs with bacterial tracheobronchitis or other inflammatory or noninflammatory pulmonary diseases.[221]
        (2) [SAA] is increased in horses with inflammatory causes of colic, including peritonitis, enteritis, colitis, and dysautonomia (grass sickness), compared to horses with strangulating or nonstrangulating noninflammatory intestinal disorders.[222]
        (3) Renal disease: In a small study, serum [CRP] was increased in dogs with naturally occurring renal disease.[223] [CRP] was negatively correlated with plasma creatinine clearance, but CRP is not small enough (M_r = 115,000) to be renally cleared in health. [CRP] was positively correlated with plasma [creatinine] and UPC ratio. These findings suggest an inflammatory state as a cause or consequence of naturally occurring renal disease in dogs.
    b. Serial monitoring

(1) Assessing response to treatment: PAPP concentrations often decrease after initiation of successful treatment and may increase with relapse, but consideration should be given to increases secondary to treatment-related injury and inflammation, e.g., repeated intramuscular injections of procaine penicillin G to horses.[224]
   (a) Dogs with steroid-responsive meningitis-arteritis had increased [CRP], [SAA], and usually [αAG] at presentation, significantly lower concentrations at remission, and concentrations WRI at resolution.[225]
   (b) In dogs, persistent increases in [CRP] and [SAA] after various orthopedic procedures are associated with complications, including infection.[226,227] However, although studies show promise for use of [CRP] to assess the postsurgical status of dogs after various types of surgery, strong evidence for such use is lacking.[228] Similarly, horses developing complications after exploratory celiotomy have an increased magnitude and duration of SAA and fibrinogen response compared to horses without complications; this might aid recognition of postsurgical complications.[184,229]

(2) Predicting relapse: Increases in post-therapy PAPP concentrations may predict relapses, as was the case for [SAA], [Hp], and [CRP] in dogs with steroid responsive meningitis-arteritis[230] and for [CRP] in a dog with *Anaplasma*-associated immune-mediated polyarthritis.[231]

(3) Guiding therapy: The return of PAPP concentrations to WRI may be useful in guiding therapy, e.g., the duration of antibiotic administration in dogs with bacterial infections (e.g., pneumonia, peritonitis, and pyometra) based on [CRP] or [SAA].[232,233]

(4) Assessing management, welfare, or fitness: Monitoring the [Hp] in dairy cows during the transition period might help recognize subclinical disease and improve management practices.[234] Assessment of [SAA] in apparently healthy horses might identify subclinical disease that reflects suboptimal welfare, including overtraining for the degree of fitness.[184]

c. Prognostication: The magnitude of one or more PAPP concentrations or changes over time may provide prognostic value, but there is often substantial overlap in values for individuals within good or bad outcome groups, making prognostication for any particular individual difficult.[184,235] Persistence of increased PAPP concentration may have more prognostic value than the magnitude of increase.[232,236]

5. Noninflammatory conditions

a. Neoplasms

(1) In general, increased PAPP concentrations may occur with a variety of neoplasms including hematologic malignancies, carcinomas, and sarcomas, but PAPPs do not all increase or increase to the same degree with different types of neoplasms, and responses vary among individuals. Mechanisms of increased PAPP concentrations include (i) production by neoplastic cells (e.g., CRP, ferritin, αAG),[237–239] (ii) inflammation induced by neoplasms, and (iii) release from nonneoplastic cells injured by the neoplastic process (e.g., ferritin release from hepatocytes).
   (a) Hematologic neoplasms: Many dogs with high-grade multicentric lymphoma, acute lymphocytic leukemia, chronic lymphocytic leukemia, and multiple myeloma have increased [CRP] and [Hp].[240–242] [αAG] may

also be increased in dogs with lymphoma.[243] Increases in [SAA] and [αAG] have been reported in cats with lymphoma.[244,245] Some horses with acute lymphocytic or myeloid leukemia had increased [SAA] or [Hp].[246] In some dogs with mast cell neoplasms, [CRP] or [αAG] are increased but [Hp] was not.[242,247] [CRP], [ferritin], or [αAG] may be increased in dogs with histiocytic sarcoma.[220,239,242]

(b) Sarcomas: In dogs with a variety of nonhematologic sarcomas of soft tissue or bone, increases in [CRP], [αAG], and [Hp] have been reported.[239,242,247,248] The [αAG] was greater than in health for some cats with sarcomas; variation within diseased and healthy groups was great.[245]

(c) Carcinomas: Increased [CRP], [SAA], [Hp], and [αAG] occur in some dogs with a variety of carcinomas,[242,248,249] and [αAG] may be increased in some cats with carcinomas.[245] In one study of dogs with mammary neoplasms, increases in [CRP], [SAA], and [Hp] were associated with metastatic disease, large neoplasms, ulceration of the mass, or concurrent disease.[249] Although [SAA], [CRP], and [Hp] were increased in dogs with benign and malignant circumanal gland neoplasms, increases were greater with the carcinomas.[250]

(2) As with inflammation, PAPPs may be useful to assess for response to therapy or relapses of neoplasms in addition to detecting septic complications. Increased concentrations of αAG and SAA in cats with large cell or small cell lymphoma decreased with therapy,[244] and the mean [αAG] of dogs with lymphoma was greater at diagnosis and relapse than after one dose of doxorubicin.[243] Increased [CRP] in 68 % of dogs with multicentric lymphoma were resolved in most dogs at remission, but variations were too great to be useful for monitoring their progress.[241]

b. Drugs

(1) Certain anthelmintic compounds (melarsonyl potassium, levamisole hydrochloride, and milbemycin oxime) can increase [Hp].[251]

(2) Phenobarbital therapy in dogs can increase [αAG].[252]

(3) Glucocorticoid therapy can cause increased [Hp] similar to Hp concentrations seen during inflammation, whereas [CRP], [SAA], and Cp activity are not increased by glucocorticoid administration.[205,253,254] Increased [Hp] may be the reason for the reported increased [α_2 globulin] in dogs.[255] Dexamethasone and cortisol (to a lesser extent) increased the release of Hp from cultured bovine hepatocytes.[256]

c. Hyperadrenocorticism: As with glucocorticoid therapy, hyperadrenocorticism can cause increased [Hp]. Mildly increased [Fbg] may be caused by hyperadrenocorticism in some dogs, but acute-phase responses (e.g., increased CRP with sepsis) may be blunted.[257]

d. Physiologic state (response to physiologic stressors)

(1) Lactation: [CRP] is reportedly increased in cattle during lactation,[258] although the contribution of subclinical inflammatory disease is not clear. The highest values were at the peak of lactation when cows were 1–4 mo pregnant. [CRP] also increased with decreases in body condition score, but the increases associated with clinically apparent inflammatory diseases were greater.

(2) Pregnancy in bitches: [CRP], [Hp], [Fbg], Cp activity, and [αAG] increase with pregnancy in bitches;[259–261] although most increases are mild, increases in

[CRP] are prominent by week 3, peaking at 4–6 wk, and often of a magnitude to support inflammatory disease.

(3) Exercise: Serum [ferritin] may increase after exercise in horses, so samples for ferritin analysis should be collected at least 2 d after strenuous exercise.[262] The exact mechanism for the increase is not established but appears to be release from tissues other than liver. Serum concentrations of several other PAPPs have reportedly increased in dogs and horses subjected to strenuous exercise, but values generally remain WRI or are only mildly increased, or appropriate RIs were not available to allow complete interpretation.[263–265]

(4) Transportation and housing changes: Transportation by road over several hours and a change in housing environments have both been associated with increases in serum [SAA] and [Hp] in cattle and with mild increases in [Hp], [CRP], and [SAA] in dogs.[266,267] The contributions of exercise and inflammation to these changes are unknown.

e. If an animal does not have inflammatory disease, serum or plasma [ferritin] tends to reflect iron storage in the animal. Thus, if iron storage is increased (e.g., hemochromatosis, hemosiderosis, or chronic hemolysis), the plasma [ferritin] might be increased (see Chapter 3).

IMMUNOGLOBULINS

The most common reason for measuring [IgG] is to determine whether there has been successful passive transfer of IgG from a mother (mare or cow) to her foal or calf via colostrum. Immunoglobulin concentrations may also be measured when evaluating congenital and acquired immunodeficiency states or when assessing potential monoclonal gammopathies.

I. Inadequate transfer of passive immunity (ITPI) (failure of passive transfer)

A. Physiologic concepts

1. Placentation in horses, cattle, and camelids prevents in utero transfer of immunoglobulins to the fetus, so neonatal horses, ruminants, and camelids must ingest colostrum soon after birth (before "gut closure") to obtain maternal immunoglobulins, especially IgG. This can be called "passive immunity." After ingestion and absorption, the half-life of maternal IgG is about 20–30 d in foals[7,268] and 20 d in calves.[269] In alpaca and llama crias, the half-life of maternal IgG has been calculated to be about 10–28 d;[270,271] both conventional and heavy chain IgG are absorbed by crias, with plasma concentrations of the latter declining more quickly.[271]
2. Neonatal foals, calves, and crias have little or no IgG in their plasma prior to ingestion of colostrum. Once exposed to antigens, neonatal foals are stimulated to produce IgG that provides protective immunity in about 10–14 d.[268] Synthesis of IgG by calves appears to occur within 2 d of birth,[272] and can occur by day 3 in foals.[7] Data were not found for llama and alpaca crias other than serial changes in serum [IgG] that indicate synthesis before 6 wk of age.[271]
3. Fetal foals, calves, alpacas, and llamas have limited ability to produce IgM, so neonates of these species have low concentrations of IgM.
4. Intestinal absorption of colostrum rapidly increases plasma [TP] and concentrations of γ and β electrophoretic fractions.[66] Most of the increase is due to increased [IgG], but

other contributing proteins include IgM, IgA, growth factors, hormones, immunoregulatory molecules, enzymes, and PAPPs (e.g., amyloid A).

5. Puppies and kittens also depend on colostrum-derived immunoglobulin for adequate transfer of passive immunity, as their placentation allows transfer of only a small amount of maternal IgG, but blood testing for ITPI is not routinely done in these species.[273]

B. Causes and consequences of ITPI
   1. Insufficient colostrum ingestion may result from neonatal factors (weakness, prematurity, or dysmaturity) or maternal factors (poor mothering or rejection of the neonate).
   2. Inadequate colostral IgG because of a decreased IgG concentration or an inadequate volume of colostrum may result from individual variability in IgG transport, premature lactation, or poor maternal nutrition or immunity. Colostral IgG may not have reactivity to all the infectious agents to which a neonate will be exposed.
   3. Failure to absorb enough ingested IgG could occur if the colostrum is ingested after "gut closure."
   4. Adequacy of transfer of passive immunity is relative to the particular environment into which the neonate is born. Inadequate immunoglobulin transfer for a particular environment increases the risk of infectious diseases (morbidity and mortality) and decreases the rate of weight gain.

C. Establishing decision limits for ITPI
   1. The endpoint for assessment of passive immunity is generally discussed as if it is dichotomous: either adequate or inadequate. In reality, transfer of passive immunity encompasses a range of IgG concentrations, creating a range of disease risks that vary with environmental and management factors. This reality is addressed in a consensus proposal that provides four-tiered individual and herd goals for post-colostral [IgG] in calves (see guidelines for calves below).[274]
   2. Decision limits are best established by determining an [IgG] above which the risk of neonatal infectious disease is minimized (or deemed acceptable) in a particular environment. Choice of decision limits affects assessment of individual neonatal risk, herd management practices, and assessments of assay performance.
   3. Choosing decision limits that yield high diagnostic sensitivity (and high negative predictive value) may be useful for screening, allowing most neonates with ITPI to be identified. Use of decision limits that yield a high diagnostic specificity (and high positive predictive value) may be useful as confirmatory tests that help reduce unnecessary medical intervention.[275]

D. When comparing the diagnostic performance of ITPI assays across studies, it is important to note whether the authors defined a positive test result as detecting "failure of passive transfer" (i.e., a test result below a specified decision threshold)[276] or as detecting "adequate passive transfer" (i.e., a test result above a specified decision threshold).[277]

E. Common IgG guidelines for recognizing ITPI: Laboratory-specific decision thresholds for [IgG] should be used when available because of assay biases (inaccuracies) that are often related to varying quality and [IgG] of calibration standards.[278,279]
   1. Calves: Blood samples are collected 1–8 d after birth, preferably at 24–48 h.
      a. Results for [IgG] have been most commonly interpreted as follows:
         (1) [IgG] > 1000 mg/dL is evidence of adequate transfer of IgG.
         (2) [IgG] = 500–1000 mg/dL is evidence of partial transfer of IgG.
         (3) [IgG] < 500 mg/dL indicates inadequate transfer of IgG.

b. More recently, the following interpretive guidelines were proposed by a group of calf experts as achievable standards to better control morbidity and mortality of dairy calves in the USA (lack of standardized IgG analysis was not addressed):[274]
   (1) [IgG] ≥ 2500 mg/dL: Excellent transfer of IgG
   (2) [IgG] = 1800–2499 mg/dL: Good transfer of IgG
   (3) [IgG] = 1000–1799 mg/dL: Fair transfer of IgG
   (4) [IgG] < 1000 mg/dL: Poor transfer of IgG
   (5) Dairy herd goal: > 40 % of a farm's calves with Excellent transfer, about 30 % and 20 % with Good and Fair transfer, respectively, and < 10 % with Poor transfer
   (6) Higher individual and herd goals may be appropriate for beef calves.

2. Foals: Blood samples are collected 12–48 h after birth and results for [IgG] are most commonly interpreted as follows.
   a. [IgG] > 800 mg/dL is evidence of adequate transfer of IgG.[268,280,281]
   b. [IgG] = 400–800 mg/dL is evidence of partial transfer of IgG.
   c. [IgG] < 400 mg/dL indicates ITPI.
3. Cria guideline: Blood samples should be collected 1–2 d after birth. [IgG] measured after 2 d can be compared to a decay curve.[270] An [IgG] < 900 mg/dL was considered inadequate transfer of IgG in 1- to 3-d-old crias based on a mortality study in Peruvian alpaca crias, but the authors suggested that inadequate transfer of IgG may have occurred with values up to 1500 mg/dL.[282] Further studies in other husbandry settings are warranted to validate or adjust these decision limits. A decision limit of 1000 mg/dL (10 g/L) is often used to identify failure to transfer passive immunity in crias.
4. Results for blood samples collected from older or ill neonates are more difficult to interpret because they are affected by the half-life of maternal IgG, a neonate's response to antigens after birth, and disease-induced loss of plasma proteins and fluids.

F. Reference laboratory assays for ITPI: These include quantitative assays of IgG and assays of total protein, globulins, or γ globulins that indirectly reflect [IgG]. The cost or turnaround time of these assays may limit their use. Anti-IgG antibodies are thought to react similarly with llama and alpaca IgG, so immunoassays developed for one species are used in the other.
1. Radial immunodiffusion (RID)
   a. Principle: IgG in patient serum diffuses in a gel containing anti-(equine IgG), anti-(bovine IgG) or anti-(camelid IgG) antibodies for 16–40 h. The diameter of a precipitant ring is proportional to the [IgG] in serum.
   b. Results: RID is considered to be a quantitative assay for measuring [IgG]. Accuracy is dependent on the quality of the reference standards (whether they verified quantitation of species-specific [IgG]). Assays for measuring bovine [IgG_1] are also available (most bovine colostral IgG is IgG_1).
   c. Although RID often serves as a gold standard for quantitation of [IgG] in neonates, commercial assays may use different calibration standards and generate different results, some overestimating [IgG].[275,279,283,284]
2. Microtiter plate ELISA
   a. Principle: Diluted patient serum is added to microtiter plate wells coated with species-specific anti-IgG antibody. Following incubation and washing, a second anti-IgG antibody conjugated to an indicator enzyme is added. After further incubation and washing, the substrate is added, followed by a quencher. Optical density is measured at a specific wavelength and compared to a standard curve of serially diluted reference material.

   b. Results: These ELISAs have been used in calves and foals, yielding quantitative results for [IgG].[285,286] Accuracy depends on the quality of reference standards (whether they verified quantitation of species-specific [IgG]).
3. Immunoturbidimetry (used in reference lab and point-of-care testing)
   a. Principle: Immunoturbidometric assays operate on the principle of precipitation of a sample's IgG by an excess of species-specific antibody. Absorbance of light by the turbid sample is measured with a spectrophotometer and is proportional to the [IgG].
   b. Results: Accuracy of these quantitative assays is dependent on the quality of the reference standards (whether they verified quantitation of species-specific [IgG]). Turbidimetric immunoassays can be run on reference and point-of-care analyzers and have been described for foals,[287,288] calves,[289] and crias.[278]
4. Serum protein electrophoresis
   a. Principle: Serum electrophoretic methods are described in Analytical Principles for Total Protein, Albumin, and Globulins, Section IV. The majority of absorbed colostral antibodies migrate in the gamma region, and thus SPE can be used to assess transfer of passive immunity. Because some immunoglobulins migrate in other regions and other proteins migrate in the gamma region, results are not the same as immunological measures of [IgG]. However, unlike immunoassays, SPE does not require use of an IgG calibration standard or curve. Comparison of pre-suckled and post-suckled serum electrophoretograms enhances assessment of the transfer of passive immunity.
   b. Results: Electrophoretic γ-globulin concentrations are positively correlated with [IgG], but they are usually lower than [IgG] determined by RID.[290–292]
5. Infrared spectroscopy
   a. Principle: Infrared spectroscopy generates infrared absorption or reflectance spectra characterizing the molecular composition of serum samples. The resulting spectra are analyzed for patterns, and algorithms based on known samples are used to quantify [IgG]. Inclusion of samples containing interfering substances (e.g., hemoglobin) during development of the diagnostic algorithms minimizes effects of interference from such substances during clinical use.[293] Various infrared spectrometers have been evaluated for assessment of [IgG] in foals,[293] calves,[294] and crias.[295]
   b. Results: Results depend on the quality of the diagnostic algorithms used to interpret the spectral bands. Based on selected decision limits and concurrent RID results, the accuracy of which depends on the quality of RID reference standards, reported diagnostic specificities for ITPI in foals, calves, and crias have been high (≥ 95 %), with moderate to high diagnostic sensitivities (71–95 %).
6. Chemical measurement of [TP] with or without calculation of [globulins]
   a. Principle: The [TP] determined by a biuret method is positively correlated with [IgG], as is the [globulins] that may be calculated from it if [albumin] is also measured; unlike [TP_{ref}], these values are not falsely increased by increases in other solutes. However, the [globulins] is typically at least twice the [IgG] because it reflects the concentration of all nonalbumin proteins (including Fbg if a plasma sample is tested). This limits diagnostic performance of [globulins] to at best moderate. Comparison of pre- and post-colostral serum better reflects transfer of passive immunity than measurement of only a post-colostral [TP].
   b. Results: Results are affected by biases in IgG, TP, and albumin assays, making transfer of conclusions to other laboratories and populations problematic. Studies of

diagnostic performance have also been limited by the analytical performance of comparative assays.

(1) Calves: A biuret [TP] of 5.2 g/dL may be a reasonable decision threshold for ITPI.[296]

(2) Foals: A [globulins] > 2.2 g/dL supports adequate transfer of IgG, and a [globulins] < 1.3 g/dL supports ITPI.[297]

(3) Crias: A [TP] > 5.5 g/dL supports adequate transfer and a [TP] < 4.0 g/dL supports ITPI.[277]

7. Gamma-glutamyl transferase (GGT) activity (see Chapter 12 for more information): GGT activity is high in bovine and canine colostrum, leading to post-suckling increases in neonatal serum GGT activity with adequate colostral intake.[296,298] In calves, assessment is best done at 1–5 d of age because of relatively rapid decreases in transferred serum GGT activity. GGT is not useful for assessing ITPI in crias or horses.

G. Point-of-care assays for ITPI (rapid screening): These assays directly or indirectly reflect [IgG] and are intended to be performed stall-side for rapid assessment and judicious intervention.

1. Latex agglutination for foals (FOALCHEK described)
   a. Principle: Latex beads coated with equine-specific anti-IgG antibodies agglutinate in the presence of IgG. Semiquantitative results are available within minutes (6-min assay time).
   b. Results: The assay is designed to detect a maximum [IgG] of > 400 mg/dL, but it can be modified by adding half the standard sample volume to detect a maximum [IgG] of > 800 mg/dL. Hemolysis does not interfere with the assay.
   c. Comments: Analytical errors may occur with errors in pipetting small volumes (2.5 or 5 µL) or variations in hematocrit when whole blood is used instead of serum. Studies suggest utility as a screening test, but not as a confirmatory test.
2. ELISA assays for foals (SNAP Foal IgG Test described)[275]
   a. Principle: The method is similar to that of the reference laboratory plate ELISA, but immunologic and chemical reagents are bonded to a paper matrix, providing for a simple, rapid, stall-side assay. These assays use serum, plasma, or whole blood and provide results in < 15 min.
   b. Results: Semiquantitative results based on color intensity are read as < 400 mg/dL, approximately 400 mg/dL, 400–800 mg/dL, approximately 800 mg/dL, or > 800 mg/dL.
   c. Comments: Studies suggest utility as a screening test, but not as a confirmatory test.
3. Immunoturbidimetry: see previous section regarding reference laboratories
4. Glutaraldehyde coagulation
   a. Principle: Glutaraldehyde promotes the formation of molecular cross-linkages between free amino groups, which are more numerous in more-basic proteins such as immunoglobulins and Fbg (the test was originally used to detect increases associated with inflammation). The degree and rapidity of coagulation (gelling) are assessed. Since there is very little IgA or IgM in neonatal sera, results reflect [IgG].
   b. Results: Various protocols are reported; all yield semiquantitative results in 1 h or less. Results have been compared with [IgG] determined using various assays.
      (1) Using a 10 % solution, strong and/or rapid coagulation was associated with [IgG] > 1200 mg/dL in calves (1 h incubation) whereas formation of a semisolid gel by 60 min reflected [IgG] of 700–1200 mg/dL.[299]

(2) In foals, a solid gel in ≤ 10 min supported adequate transfer of IgG (RID [IgG] > 800 mg/dL); lack of a solid gel by 1 h had high diagnostic sensitivity and specificity for ITPI.[300,301]

(3) In one study of this test in llama crias, there was reportedly no significant correlation between glutaraldehyde coagulation and [IgG] by RID.[302]

c. Comments: Although current assay directions indicate that whole blood is an acceptable sample, comparison data for sample types were not found. Use of serum allows recognition of hemolysis and avoids Fbg, each of which can cause overestimation of [IgG]. Results of one commercial glutaraldehyde test using whole blood were not considered reliable for calves given a weak association between glutaraldehyde coagulation time and [IgG] based on a RID assay.[303]

5. Precipitation by salt solutions

a. General principle: Solutions of high ionic strength decrease the solubility of proteins to varying degrees depending on the added ions, the concentration of added ions, and the structure of the proteins (size, shape, and charged groups). This allows for somewhat selective precipitation of IgG from neonatal sera. Testing is relatively inexpensive and quick using commercial kits or solutions prepared in-house.

b. $ZnSO_4$ turbidity

(1) Protocols vary regarding single or multiple concentrations of $ZnSO_4$ solution and incubation times (30–60 min). At a constant [$ZnSO_4$], a greater turbidity corresponds to a greater [IgG]. The degree of turbidity increases with greater $ZnSO_4$ concentrations.[304] Turbidity can be assessed visually for semiquantitative results or spectrophotometrically with a standard curve for quantitative results.[292]

(2) Results

(a) In calves, high turbidity (obscures newsprint) was associated with [IgG] > 1600 mg/dL (using a 208 mg/L $ZnSO_4$ solution), consistent with adequate transfer of IgG.[299]

(b) In foals, visual turbidity occurred even when the [IgG] was 400–500 mg/dL and indicative of ITPI (using a 250 mg/L $ZnSO_4$ solution),[291] consistent with a reported low positive-predictive value for detecting ITPI.[275]

(c) $ZnSO_4$ turbidity did not reliably detect ITPI in llama crias;[302] however, turbidity was assessed using a bovine IgG standard curve.

(3) Comments: Turbidity is affected by temperature and pH (stock solutions need to be sealed). Hemolysis (hemoglobin) falsely increases the measured [IgG] if turbidity is assessed by spectrophotometry at 660 nm; Hgb-induced increments were reported to be about 200 mg/dL at 1 % hemolysis and 1300 mg/dL at 5 % hemolysis.[292]

c. Sodium sulfite turbidity (Na_2SO_3 precipitation)

(1) Greater concentrations of sulfites have greater capability of precipitating IgG at lower concentrations.

(2) Results: Various protocols are reported using single (18 %) or serial (18 %, 16 %, and 14 %) concentrations of Na_2SO_3 and different incubation times (15 min to 1 h) to produce semiquantitative results easily and quickly.

(a) Estimated calf [IgG] based on precipitation in one (18 %), two (18 % and 16 %), or all three of the solutions ostensibly indicative of

[IgG] < 500 mg/dL, 500–1500 mg/dL, and > 1500 mg/dL, respectively, aligned relatively well with [IgG] decision thresholds in one study,[299] and had high diagnostic sensitivity and specificity (≥ 90 %) in another study when using the absence of precipitation in all three concentrations as an indication of ITPI.[285]

(b) The assay did not work well for foal serum, as precipitation underestimated [IgG] in 7 of 20 samples and overestimated it in 1 of 20.[305]

(c) In one study, traditional 18 %, 16 %, and 14 % solutions did not reliably detect ITPI in alpaca crias.[277] In the same study, a commercially available kit also underestimated [IgG], but an empirical decision limit yielded an accuracy of 89 % for identifying crias with adequate transfer of passive immunity ([IgG] > 1000 mg/dL based on RID).

(3) Comments: Use is limited by uncertain accuracy of comparative RID assays, lack of agreement between RID-measured [IgG] and estimates based on precipitation results, and lack of alignment between designated [IgG] estimates of each solution and the decision thresholds used to interpret results.

d. Ammonium sulfate precipitation[306]

(1) The amount of protein precipitate formed after a 2-min incubation of a 40 % ammonium sulfate solution with an equal volume of serum can be assessed by transferring the vortexed suspension to a microhematocrit tube and measuring the "immunocrit" (volume percentage of precipitate) after microhematocrit centrifugation.

(2) Results: The "immunocrit" correlated with an AGE assessment of immunoglobulin concentration in foals, and an "immunocrit" < 9.5 % suggested IPTI, defined by < 800 mg/dL of serum immunoglobulins.

(3) Comments: In this study, AGE fractionation and γ-globulin concentrations were not provided. Neither ammonium sulfate precipitation nor AGE is specific for immunoglobulins or IgG.

6. Refractometric serum total protein concentration [sTP_{ref}]

a. Principle: A neonate's postcolostral [sTP_{ref}] is compared to a decision threshold for ITPI or, if precolostral samples are first tested, the change in [sTP_{ref}] from precolostral to postcolostral samples (1 d after ingestion) is compared to the increases expected with adequate absorption of immunoglobulins from colostrum.[284] Because dehydration nonselectively increases the concentration of blood solutes, [sTP_{ref}] overestimates passive transfer in dehydrated neonates. Decision limits are higher if [TP_{ref}] is assessed in heparinized plasma, and results may be less predictive of [IgG].[307]

b. Results

(1) Calves

(a) Based on a goal of [IgG] > 1000 mg/dL and a meta-analysis of 11 studies with a total of 1814 calves using RID or turbidimetric immunoassays as reference methods, [sTP_{ref}] < 5.2 g/dL was considered the best indication of ITPI (mean diagnostic specificity of 89 %), and [sTP_{ref}] > 5.5 g/dL was considered the best indication to exclude ITPI (mean diagnostic sensitivity of 88 %).[308] In beef calves, [sTP_{ref}] ≤ 5.1 suggested [IgG] < 1000 mg/dL, whereas [sTP_{ref}] ≤ 5.7 suggested [IgG] < 2400 mg/dL.[309]

(b) The following guidelines for [TP] (presumable [sTP_{ref}]) in dairy calves were proposed by a group of calf experts who considered these cutoffs equivalent to their [IgG] cutoffs:[274]
(i) [TP] ≥ 6.2 g/dL: Excellent transfer of IgG
(ii) [TP] = 5.8–6.1 g/dL: Good transfer of IgG
(iii) [TP] = 5.1–5.7 g/dL: Fair transfer of IgG
(iv) [TP] < 5.1 g/dL: Poor transfer of IgG
(v) Others reported that [sTP_{ref}] cutoffs of 5.0 and 5.8 g/dL best estimated [IgG] cutoffs defining these Poor and Excellent categories.[310]

(c) Compared to [sTP_{ref}] in calves receiving natural bovine colostrum, [sTP_{ref}] in calves receiving colostrum replacer was substantially lower for a given [IgG] and did not correlate as well with [IgG].[311] Therefore, a lower [sTP_{ref}] cutoff may be appropriate for calves that received colostrum replacer.

(2) Foals: Correlations of RID-determined [IgG] with [sTP_{ref}] or the incremental increase in [sTP_{ref}] after colostral absorption are only moderate (r = 0.79–0.86), thus limiting diagnostic performance for detecting ITPI. Based on several studies, a [sTP_{ref}] > 6.0 g/dL is a strong indication of adequate transfer of IgG ([IgG] > 800 mg/dL), and a [sTP_{ref}] < 4.2–4.6 g/dL is very supportive of ITPI ([IgG < 400 mg/dL).[275,312–314]

(3) Crias: RID results for serum [IgG] in crias have been compared to what authors have called serum "total solids" concentration but what was likely [sTP_{ref}] based on the types of refractometers or the units used for reporting results.[277,302] RID and refractometric results either lacked a significant correlation or correlated moderately, the latter with a RID assay that appeared to be inaccurate given results for [IgG] > 3–4 g/dL in samples from several crias. Utility of refractometry for evaluation of passive transfer in crias has not been demonstrated.

c. Brix refractometry: Brix refractometers have been used to assess for ITPI, but they were designed to measure the sucrose concentration of aqueous solutions in °Bx or Brix %, where 1 °Bx (1 Brix%) indicates 1 g/dL (1 % by mass) of sucrose. The scale is not calibrated for proteins in serum. However, the performance of Brix refractometry for assessing ITPI is similar to that of [sTP_{ref}] using empirical cutoffs.

(1) Calves: Reported cutoffs for ITPI ([IgG] < 1000 mg/dL) vary from 8.1 % to 8.7 %, with values ≥ 9.4 % supporting excellent transfer of IgG ([IgG] ≥ 2500 mg/dL).[274,307,309,310]

(2) Foals: Reported cutoffs for [IgG] ≤ 400 or 800 mg/dL are 7.8–7.9 %.[307,313]

H. Each of the methods of measuring or estimating [IgG] in foal, calf, or cria sera has its advantages and disadvantages. The most accurate test may have limited clinical applicability, whereas the most convenient or inexpensive test may be prone to unreliable results.

II. Immunoglobulin deficiency

A. Immunoglobulin concentrations can be measured to detect or confirm the presence of congenital immunodeficiencies. Age-specific RIs are important when interpreting concentrations of immunoglobulins in young animals.

B. Analytical concepts

1. Documenting a marked decrease in an immunoglobulin concentration requires species-specific assays that have an appropriate analytical range, i.e., the capability of

accurately measuring concentrations below lower reference limits. Typically, these assays are RID assays, but other immunologic assays can be used. Because immunoglobulins in healthy animals are responsible for almost half of the total [globulins], a complete absence of immunoglobulins might cause hypoglobulinemia. However, there are many other more common reasons for hypoglobulinemia (see Hypoglobulinemia above).

2. Because most of the γ-globulin fraction of SPE is composed of IgG, and therefore most γ globulins are IgG molecules, an IgG deficiency can be suspected when there is hypogammaglobulinemia. However, the suspicion should be confirmed with a species-specific quantitative assay for IgG or IgG subclasses.
3. Because IgA and IgM represent only a small amount of the β or γ globulins in health, deficiencies in IgA and IgM cannot be detected by routine SPE.

C. Congenital immunodeficiencies causing decreased immunoglobulin concentrations
1. Horses[315,316]
   a. Severe combined immunodeficiency: This is an autosomal recessive disorder occurring mostly in Arabian and Appaloosa foals; it was reported in one Caspian foal. Affected foals do not produce functional T or B lymphocytes, and thus they succumb to infections shortly after maternal antibodies obtained from colostrum are degraded (~6 d for IgM, and ~2–3 wk for IgG). Other than the absence of IgM in presuckle serum, other diagnostic features include persistent lymphopenia and marked lymphoid hypoplasia in lymphoid organs. Affected horses have a mutated gene for a DNA-dependent protein kinase required for production of functional antigen receptors; the mutation can be detected by a PCR-based assay.
   b. Primary agammaglobulinemia in thoroughbreds, standardbreds, and quarter horses: Affected foals do not produce IgG, IgA, or IgM because they lack B lymphocytes, but they do have functional T lymphocytes. Presuckle serum lacks immunoglobulins, and maternal IgG and IgM obtained from colostrum disappear over time to yield agammaglobulinemia. All reported cases were colts, suggesting, but not confirming, an X-linked disorder.[317,318]
   c. Selective IgM deficiency: Affected foals (mostly Arabian and quarter horses) have a marked decrease in serum [IgM] but do produce other immunoglobulins. B-lymphocyte concentrations are not decreased.
   d. Selective IgG deficiency: Affected foals have a marked decrease in serum [IgG] but do produce other immunoglobulins.
   e. Common variable immunodeficiency: Affected horses (2–19-yr-olds of several breeds) had low serum [IgG], [IgM], and eventually [IgA] with persistent B-cell lymphopenia and absence of B cells and lymphoid follicles in lymphoid tissue.[319]
   f. Anemia/immunodeficiency syndrome (Fell pony syndrome): Some Fell and Dales ponies have a fatal autosomal recessive disorder in which there are defects in postnatal erythropoiesis and production of B lymphocytes that cause severe anemia, B-lymphocyte deficiency, and decreased serum [IgM] and [IgG] (after waning of colostral immunoglobulin).[320–323] If IgG is detected in serum, it is maternal IgG. The genetic mutation can be detected with a PCR-based assay.
   g. Transient hypogammaglobulinemia of neonates: Affected foals have delayed production of IgG and IgA, which causes low serum concentrations once colostral immunoglobulins have cleared at about 2–3 mo of age and before autologous production is sufficient by about 5–7 mo of age.[324]

2. Cattle[315]
   a. Severe combined immunodeficiency in an Angus calf: IgM and IgA were not detected in the serum of the affected 6-wk-old calf; a very low [IgG] was considered to be of maternal origin. The calf lacked lymphocytes in its thymus, lymph nodes were not detected, and its spleen was small.
   b. Selective IgG_2 deficiency: About 1–2 % of red Danish cattle lack detectable IgG_2, but they do have other immunoglobulins. An IgG_2 deficiency has also been reported in a Holstein heifer.[325]
3. Dogs[315]
   a. Severe combined immunodeficiency
      (1) Jack Russell terriers (autosomal recessive): Affected dogs lack B and T lymphocytes and thus cannot produce immunoglobulins. They have severe lymphopenia and generalized lymphoid hypoplasia to aplasia.[326]
      (2) Basset hounds and Cardigan Welsh corgis (X linked): Affected dogs lack IgG and IgA in serum but the [IgM] is WRI. The dogs have a marked reduction in CD8 lymphocytes and are lymphopenic. The defective mutations are different in the two breeds.
   b. Selective IgM deficiency in Doberman pinschers: Affected dogs had increased serum [IgA], decreased [IgG], and very low [IgM].
   c. Selective IgA deficiency has been reported in a variety of breeds including German shepherds, shar peis, and beagles in an inbred colony.[327]
   d. Immunoglobulin deficiency in Weimaraners: Affected pups have chronic infections, decreased serum [IgG], and variably decreased [IgA], [IgM] or both.[328,329]
   e. Suspected immunodeficiencies in *Pneumocystis*-infected dogs including miniature dachshunds, Cavalier King Charles spaniels, a Pomeranian, and a shih tzu: The [IgG] in affected 9–12-mo-old miniature dachshunds was lower than in age- and breed-matched dogs, but not statistically significantly.[330] The Cavalier spaniels had low [IgG] compared to other Cavaliers, but increased [IgM] during active infection.[331] The 20-mo-old Pomeranian had decreased serum [IgG], mildly decreased [IgA], and low-normal [IgM] compared to adult RIs.[332] The 1-yr-old male shih tzu had serum IgG and IgA concentrations at the lower reference limits when higher concentrations would have been expected in response to disease; a nonsense mutation was found in the dog's CD40 ligand gene and predicted to cause impaired T- and B-cell immune responses.[333]
   f. Suspected immunodeficiency in Rottweilers: Each of seven puppies from one litter had a decreased [IgA] and each of six of these had a low to low-normal [IgG].[334]
4. Llamas[335]
   a. Juvenile llama immunodeficiency syndrome is characterized by wasting or failure to grow, low-grade chronic infections that respond poorly to treatment, hypoalbuminemia, and mild nonregenerative anemia or marked regenerative anemia when infected with "*Candidatus* Mycoplasma haemolamae."
   b. Most affected llamas had decreased [IgG] compared to healthy juvenile or adult llamas, poor response to *Clostridium perfringens* C toxoid, lymphopenia of B lymphocytes (prior to clinical signs and not present in parents),[335] and moderate to marked paracortical lymphoid depletion in lymph nodes with decreased secondary follicles. The findings suggest a failure of B-lymphocyte development due to an autosomal recessive genetic defect.[336]

D. Transient hypogammaglobulinemia of neonates: As noted in Section C.1.g, foals may have a delayed onset of immunoglobulin synthesis causing hypogammaglobulinemia at a few months of age; this was reported in an Arabian foal and a thoroughbred foal, each without lymphopenia.[317,337] A similar condition was reported in dogs[324] and in a Simmental heifer. The cause or causes of these disorders is unknown, as is the cause of transient hypogammaglobulinemia of infancy in people.

III. Immunoglobulin excess

A. Hyperglobulinemia is usually caused by a chronic infection or dehydration, but it may be due to excessive production of an immunoglobulin by neoplastic B lymphocytes or plasma cells. If not caused by hemoconcentration, SPE may provide evidence for either a chronic inflammatory state or lymphoid neoplasm. When a monoclonal gammopathy is suspected, it is typically more important to characterize the immunoglobulin than to measure immunoglobulin concentrations.

B. An earlier section (Hyperproteinemia, Section II.B) of this chapter described diagnostic methods that can be used to help differentiate an inflammatory oligoclonal gammopathy from a lymphoid neoplastic gammopathy. Most of those diagnostic assays target the heavy chain of the immunoglobulin class or subclass and do not prove clonality. Documenting that a gammopathy is caused by only one IgG subclass, or IgM, or IgA would be better evidence of a monoclonal state because it would be unlikely that the concentration of only one of these would be increased in an inflammatory state. Establishing that a neoplastic clone is producing a single antigen-specific immunoglobulin is nearly impossible.

C. Most monoclonal gammopathies are diagnosed by association, i.e., finding a probable monoclonal gammopathy by SPE and a concurrent lymphoid neoplasm (e.g., lymphoma, lymphoid leukemia, plasmacytoma, or myeloma). Most polyclonal gammopathies are diagnosed by finding a probable polyclonal gammopathy by SPE and a concurrent chronic inflammatory state, most commonly caused by a chronic infection. These conclusions become more difficult and uncertain when these typical associations are not found, e.g., possible monoclonal gammopathy in an animal with a chronic infection. Then, more definitive characterization of the immunoglobulins may establish the pathogenesis for the gammopathy.

PATTERNS OF PROTEIN CONCENTRATIONS IN MAJOR DYSPROTEINEMIAS

Results for [TP], [albumin], and [globulins] are routinely interpreted together, because the patterns of protein changes provide more diagnostic information than each abnormality alone. Data for PAPPs, including plasma [Fbg], may be available concurrently. These findings may be enough for patient management, or they may lead to further testing, including serum protein electrophoresis, immunoelectrophoresis, or measurement of immunoglobulin concentrations. See Table 7.10 for typical patterns of [TP], [albumin], and [globulins] in major dysproteinemic conditions.

COLLOID OSMOTIC PRESSURE (COP) (ONCOTIC PRESSURE)

I. Physiologic processes and concepts

A. COP is also called oncotic pressure; the prefix *onco-* comes from the Greek for "bulk" or "swelling," which, in this context, refers to the swelling of spaces when fluid enters them because of the presence of colloidal particles. The prefix is also commonly used in the context of oncology, the study of neoplastic masses.

Table 7.10. Typical protein patterns for major dysproteinemic conditions (these conditions may be present without a detected dysproteinemia)[24]

Condition	[TP][a]	[Albumin]	[Globulins]	A:G ratio
Hemoconcentration	↑–↑↑	↑–↑↑	↑–↑↑[b]	WRI
Established inflammation	↑–↑↑↑	↓–WRI	↑–↑↑↑[bc]	↓–↓↓
Plasma cell myeloma (secretory)	↑–↑↑↑	↓–WRI	↑–↑↑↑[d]	↓–↓↓
Acute blood loss	↓–↓↓	↓–↓↓	↓–↓↓	WRI
Protein-losing nephropathy	↓↓–WRI	↓–↓↓↓	WRI[ef]	↓
Protein-losing enteropathy	↓–↓↓↓	↓–↓↓	↓↓–WRI	↓–WRI
Protein-losing dermatopathy[g]	↓–↓↓	↓–↓↓	↓↓–WRI	↓–WRI
Peritonitis, pleuritis, vasculitis, sepsis (plasma protein loss)[h]	↓–↓↓	↓–↓↓	↓–WRI	↓–WRI
Hepatic insufficiency	↓–WRI	↓–↓↓	↓–↑	↓↓–WRI
Malabsorption or maldigestion	↓–↓↓	↓–↓↓	↓–WRI	↓–WRI
Cachectic states	↓	↓	↓–↑	↓–WRI
Lymphoid hypoplasia or aplasia[i]	↓–WRI	WRI	↓–WRI	WRI–↑
ITPI[i]	↓–WRI	WRI	↓	WRI–↑
Hemodilution	↓	↓–WRI	↓–WRI	WRI

[a] [TP] based on a chemical method; hyperproteinemia based on [TP_{ref}] may be falsely increased by interferents.
[b] Plasma [Fbg] may be increased.
[c] A polyclonal gammopathy is common on SPE, but oligoclonal or apparent monoclonal gammopathies may occur; concentrations of various PAPPs may be increased.
[d] Monoclonal gammopathy on SPE, including atypical patterns (see text)
[e] Hyperglobulinemia may occur with some disorders, e.g., leishmaniasis.
[f] Concentrations of globulins other than α_2-macroglobulin may be decreased; only with extreme PLN will [globulins] be decreased, but they may be in the lower portion of the RI in some cases.
[g] Hyperglobulinemia and perhaps hyperproteinemia could develop with associated inflammation.
[h] Hypoproteinemia with acute extravasation of plasma and subsequent hemodilution. Otherwise, inflammatory dysproteinemia may be the predominant pattern.
[i] Values for neonates must be interpreted by using neonatal RIs.
Notes: Pattern variations exist and are expected with concurrent conditions. Age and breed variations should be considered when interpreting protein concentrations (see footnotes in Tables 7.4 and 7.6).

B. Hydraulic pressure gradients and COP gradients force the movement of protein-poor fluids out of and into capillaries as described by Starling's law (Figure 7.7 and Eq. 7.2). In health, these gradients are important for the maintenance of blood volume. In edematous, transudative, and exudative disorders, alterations in the gradients contribute to the movement of plasma H_2O into interstitial spaces (e.g., see Chapter 19, General Concepts and Definitions, V.).

$$\text{Pressure gradient} = \Delta \text{ hydraulic pressure} - \Delta \text{ oncotic pressure} = \left(P_{capillary} - P_{interstitial\ fluid}\right) - \left(\pi_{capillary} - \pi_{interstitial\ fluid}\right) \quad (7.2)$$

C. COP is caused by concentrations of colloidal solutes or particles. *Colloidal particles* in blood are macromolecules that are too small to settle out due to gravity but too large

to permeate intact vascular membranes. They are generally 1 nm to 1 μm in diameter, with M_r > 30,000, though colloidal properties are affected by factors other than size (e.g., surface area). In normoproteinemic plasma, albumin contributes about 75–80 % of total COP.

1. COP develops whenever two fluids with different concentrations of colloidal particles are separated by a semipermeable membrane. In vivo, capillary walls act as semipermeable membranes.
2. Of the total 25 mmHg COP in human capillary plasma, about 17 mmHg is directly related to plasma proteins and about 8 mmHg is due to the Gibbs–Donnan equilibrium.[339]
   a. The major concepts of the Gibbs–Donnan equilibrium are illustrated in Figure 7.8. The proteins "trapped" in the intravascular space have a negative charge. For electroneutrality, there must be enough diffusible cations (e.g., Na^+) in the plasma to balance the negative charges of the proteins. In the Gibbs–Donnan equilibrium, the product of the major diffusible cation charge concentration and major diffusible anion charge concentration of the plasma equals the product of the major diffusible cation charge concentration and major diffusible anion charge concentration of the interstitial fluid (Eq. 7.3 and Figure 7.8).

$$[Na^+]_{plasma} \times [Cl^-]_{plasma} = [Na^+]_{interstitial\ fluid} \times [Cl^-]_{interstitial\ fluid}$$
$$[major\ cations^+]_p \times [major\ anions^-]_p = [major\ cations^+]_{if} \times [major\ anions^-]_{if} \quad (7.3)$$

   b. This protein-induced imbalance of ions causes a greater osmolality in blood and augments the direct COP. In health, plasma proteins contribute about 0.9 mmol/kg to plasma osmolality, and the Gibbs–Donnan effect contributes about 0.4 mmol/kg.[339]

II. Analytical Concepts

A. The osmotic effect of proteins is used to measure COP with a colloid osmometer as illustrated in Figure 7.9.

B. Blood, plasma, and serum samples can all be used. Anticoagulation with EDTA or heparin are appropriate, but not citrate because it would cause dilution by a factor of about 10 %. Compared to plasma and serum, blood has the disadvantage of masking sample hemolysis which may falsely increase the COP, and blood may contain unseen small clots that can plug tubing; however, blood allows for more immediate testing in emergency settings.

C. Very little or no difference in COP is detected with the different sample types.[340–342] Blood cells are not colloidal particles so they do not contribute directly to COP; however, slight changes in blood COP (1–2 mmHg) may occur with marked changes in blood pCO_2 or $[HCO_3^-]$.[343] Fbg contributes to COP of blood or plasma but not of serum.

D. Units: 1 mmHg = 1 torr; 1 mmHg × 133.322 = 1 pascal

E. Interferences

1. Hgb in the sample increases the COP because Hgb is a colloidal particle. In a small study, Hgb increased COP at a rate of nearly 4 mmHg per every 1 g/dL of Hgb.[344] This falsely increases COP if the Hgb is from in vitro hemolysis. Hemoglobinemia from in vivo hemolysis would also increase COP, but not falsely.

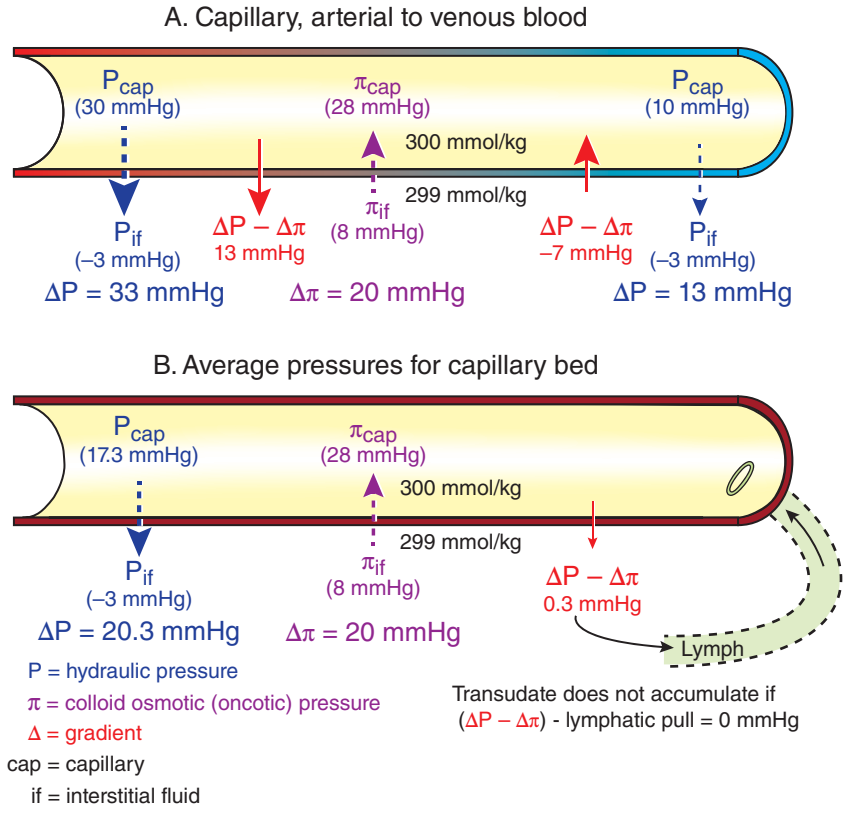


Figure 7.7. Illustration of fluid movements because of Starling's law in a typical bed of continuous-type capillaries.

A. Typical pressures in a capillary bed, from arterial to venous end
- Hydraulic pressures in health
  - The plasma hydraulic pressure in the arterial side of the capillary bed is much greater than the hydraulic pressure in the interstitial fluid. The difference in the hydraulic pressure is called the hydraulic pressure gradient (Eq. 7.2). A typical hydraulic pressure gradient (ΔP) on the arterial side of the capillary is about 33 mmHg.
  - The plasma hydraulic pressure in the venous side of the capillary bed is greater than the hydraulic pressure in the interstitial fluid. A typical ΔP on the venous side of the capillary is about 13 mmHg.
  - The hydraulic pressure in the interstitial fluid is about –3 mmHg. The negative pressure is created by the actions of valves in the lymphatic vessels and pressure changes in vena cava vessels.
- COPs (oncotic pressures) in health
  - The plasma COP is greater than the interstitial COP because the plasma [total protein] is greater than the interstitial fluid [total protein]. The [total protein] essentially does not change in the capillary beds, and thus the COP gradient (Δπ) remains the same from the arterial side to the venous side of the capillary bed. For most capillaries, the Δπ is near 20 mmHg.
  - Most of the COP (both in plasma and in interstitial fluid) is due to albumin, but globulins do contribute. See the text for more information about plasma COP.
  - The difference between the ΔP and Δπ (i.e., ΔP–Δπ) is a major factor that determines the rate of flow of fluid out of and into capillaries. On the arterial side, the difference is about 13 mmHg, and thus fluid leaves the capillary and enters the interstitial space. On the venous side, the difference is about –7 mmHg and thus fluid leaves the interstitial space and enters the capillary.

2. Plasma expanders (e.g., hetastarch, dextran, and succinylated gelatin) increase COP.[34] In samples containing hetastarch and dextran, the COP does not represent the concentration of colloidal proteins. Succinylated gelatin is a modified protein and increases the [TP], but COP increases to a disproportionately greater degree.

F. Comments: Various formulas have been proposed for estimating or calculating COP by using measured concentrations of plasma proteins. Because of differences in pH or concentrations of each plasma protein among individuals of a species and among different species, these formulas may not provide a reliable estimate of plasma COP, particularly in disease.[342,345–348] Also, the formulas are not useful after treatment with synthetic colloids.

III. Increased COP

A. By itself, an increased COP is not a common diagnostic problem for two reasons: (i) it is typically due to hyperproteinemia, and this condition is more easily detected by other means, and (ii) COP is rarely measured in samples that might have an increased COP.

B. Increased COP can occur with increases in colloidal particles that are typically absent from plasma.

1. Increased plasma or serum Hgb concentration
   a. In plasma, free Hgb from in vivo or in vitro hemolysis can increase plasma COP.
   b. Infusion of Hgb-based oxygen carriers (e.g., Oxyglobin from Biopure Corp., 1984–2009) quickly increases plasma COP. In one study, the Hgb-based oxygen carrier had a COP about twice that of canine plasma (43 mmHg vs. 20 mmHg). Infusion increased the plasma COP quickly to about 36 mmHg, but the COP returned to baseline values after 3 h.[349]
2. Infusion of plasma expanders such as hetastarch can increase COP.[35,350,351] but it depends on the administration protocol and state of the animals. When plasma expanders are administered to animals that have a decreased COP because of marked hypoproteinemia, it is unlikely that enough hetastarch would be given to increase the plasma COP value to the RI. When given to dogs at a rate of 20 mL/kg body weight over 4 h, the plasma COP values typically increased 2–4 mmHg but returned to baseline values by 12 h.[344]

Figure 7.7. *continued*

B. Average pressures for a typical continuous-type–capillary bed

- Even though the ΔP on the arterial side is much greater than the ΔP on the venous side, the net difference in tissues is only about 20.3 mmHg. The difference between the ΔP and Δπ is only 0.3 mmHg. Most fluid returns to the capillaries because of two factors: (i) there are more venous capillaries (entering venules) than arterial capillaries (leaving arterioles), and (ii) the venous capillaries are more permeable than arterial capillaries.[338]
- Fluid not returned directly to blood through capillary walls is transported to blood via lymphatic vessels. If the lymphatic system removes the interstitial fluid, a transudate does not accumulate.
- Even though the COP is greater in plasma than in interstitial fluid, there is very little difference in the osmolalities of the fluids (300 mmol/kg vs 299 mmol/kg) because proteins contribute very little to total osmolality, and the concentrations of major solutes (i.e., electrolytes, glucose, and urea) are nearly the same in the two fluids (see Chapter 9). The Gibbs–Donnan effect (Figure 7.8) creates differences in the osmolality.

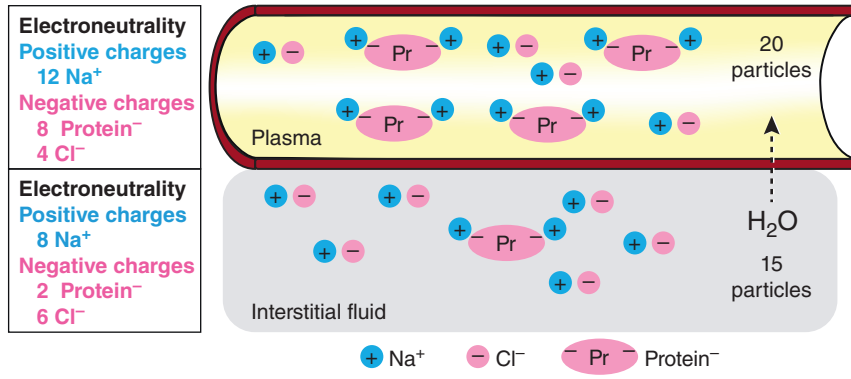


Figure 7.8. Conceptual representation of the Gibbs–Donnan equilibrium. The protein concentration in plasma is greater than in interstitial fluid, and the net charge of plasma proteins is negative. The wall of a typical continuous-type capillary is nearly impermeable to proteins but is permeable to Na^+ and Cl^- (and other electrolytes). Movement of cations and anions is controlled by concentration and charge gradients. Negatively-charged proteins attract diffusible cations (e.g., Na^+) to plasma, while diffusible anions (e.g., Cl^-) are repelled toward interstitial fluid. At equilibrium, electroneutrality occurs on each side of the capillary wall; in this representation, 12 cation charges are balanced by 12 anion charges in the plasma, and eight cation charges are balanced by eight anion charges in interstitial fluid (each protein is a divalent anion in this simplification). Because of the negatively charged plasma protein molecules, there are more diffusible cations and fewer diffusible anions in plasma (12 and 4, respectively) than in interstitial fluid (8 and 6, respectively). Osmolality, determined by the number of solute particles (molecules or ions/kg H_2O), is greater in the plasma (20 particles) than in interstitial fluid (17 particles), thus creating an osmotic gradient that promotes the flow of H_2O from interstitial fluid to plasma. This COP is generated by the presence of nondiffusible proteins, which causes a greater osmolality in plasma than interstitial fluid (Gibbs–Donnan effect). The Gibbs–Donnan effect increases with the square of the protein charge. Since the average protein molecule has more than one negative charge, the Gibbs–Donnan effect is exponentially greater at greater protein concentrations (not linear).

IV. Decreased COP
   A. Decreased COP values result from hypoproteinemia.
   B. The primary value of measuring COP is for the clinical management of severely hypoproteinemic animals. Because marked hypoproteinemia (especially if an acute onset) can result in the movement of water from blood to cause hypovolemia and edematous states, plasma or plasma expanders (such as hetastarch) may be administered to increase blood COP and thus reduce the pathologic consequences of hypoproteinemia. However, animals suffering from sepsis, systemic inflammatory response syndrome, or vasculitis may have increased vascular permeability that contributes to hypoalbuminemia (and hypoproteinemia) and also allows administered colloids to enter extravascular tissue and contribute to edema.[153]
      1. Hetastarch administration increases COP, but the duration of its effects depends on two major factors: (i) the rate at which the hetastarch is degraded by the animal and (ii) the permeability of capillaries to hetastarch.
      2. The degree to which hetastarch increases the COP is somewhat less than one might expect. When hetastarch is first given, it quickly increases the plasma COP and thus alters the COP gradient in capillary beds. As a result, more water is pulled back into the

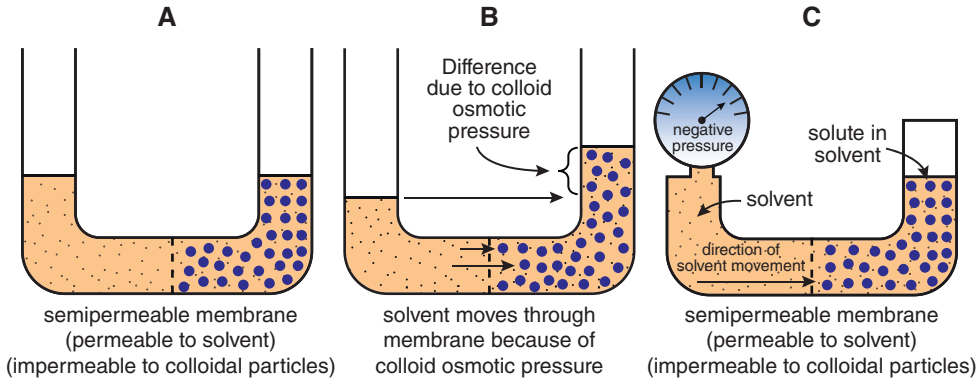


Figure 7.9. Schematic representation of the principles of colloid osmometry.

A. Two fluids are present in an open-tube system, a crystalloid fluid such as isotonic saline and a similar fluid that also contains large solute colloidal particles (e.g., proteins). The fluids are initially separated by a semipermeable membrane – a membrane that is permeable to the solvent and its small solute particles but not permeable to the large colloidal particles. The presence of large colloidal particles on one side results in a higher osmolality (see the Gibbs–Donnan effect in Figure 7.8) and thus an osmotic gradient. This gradient creates osmotic pressure, thus the term *colloid osmotic pressure*. The COP initiates the movement of solvent towards the colloidal particles.

B. When the system's pressures equilibrate, solvent has moved to the fluid that contained the colloidal particles. The difference in the fluid levels is primarily due to the COP that was created by the colloidal particles. Other factors (such as bulging of the membrane in this open system and gravity) also affect the difference.

C. In the closed system of a colloid osmometer, the effects of membrane bulging and gravity are reduced. However, the same general principles apply, and the colloidal particles attract the fluid. The COP is measured by a pressure transducer.

plasma to expand blood volume, but this concurrently reduces [TP]. Thus, the protein contribution to the measured COP is reduced.

3. When there is a marked decrease in COP, administration of plasma has two major advantages over administration of hetastarch: (i) the circulating half-life of most plasma proteins (especially albumin) is much greater than that of hetastarch, and (ii) the increase in COP is due to plasma proteins, and thus effects can be monitored by either COP or plasma protein concentrations.

C. When an animal's blood has a decreased COP, the animal is prone to develop edema or a transudate. However, other factors also influence the extravasation of fluid.

1. Extravasation of a small amount of fluid to the interstitial space increases the hydraulic pressure in the interstitial space, thus limiting additional extravasation.
2. Extravasation of protein to the interstitial space is less than the extravasation of H_2O (unless permeability is altered by inflammation), so the protein concentration in the interstitial fluid decreases and there is an increased COP gradient that limits additional extravasation.
3. The lymphatic system can greatly increase its drainage of interstitial fluid in some tissues. Thus, even though fluid may be leaving the plasma faster, it may not accumulate in the interstitial space.
4. Retention of Na^+ and H_2O is largely responsible for most transudative states (e.g., cirrhosis, PLN, and heart failure) (see Chapter 19).

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 8

URINARY SYSTEM

Michael A. Scott and Steven L. Stockham

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Table 8.1. Abbreviations and symbols in this chapter	
[x]	x concentration (x = analyte)
$1,25(OH)_2D$	1,25-Dihydroxycholecalciferol
AAP	Alanine aminopeptidase
ADH	Antidiuretic hormone (arginine vasopressin)
ALP	Alkaline phosphatase
AQP	Aquaporin
ATPase	Adenosine triphosphatase
Bc	Conjugated bilirubin
Bu	Unconjugated bilirubin
Ca^{2+}	Calcium
Crt	Creatinine
DCT	Distal convoluted tubule
ELISA	Enzyme-linked immunosorbent assay
FE	Fractional excretion
fCa^{2+}	Free ionized calcium (not bound or complexed)
fMg^{2+}	Free ionized magnesium (not bound or complexed)
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
GGT	γ-Glutamyltransferase
HCO_3^-	Bicarbonate
hpf	High-power field (400× magnification = 40× objective and 10× ocular)
LoH	Loop of Henle
lpf	Low-power field (100× magnification = 10× objective and 10× ocular)
M_r	Relative molecular mass
NAG	N-acetyl-β-D-glucosaminidase
NH_3	Ammonia
NH_4^+	Ammonium
PD	Polydipsia
PO_4	Phosphate including PO_4^{3-}, HPO_4^{2-}, or $H_2PO_4^-$
PCT	Proximal convoluted tubule
PTH	Parathyroid hormone
PU	Polyuria
RI	Reference interval
RBC	Red blood cell (erythrocyte)
RPF	Renal plasma flow
SD	Standard deviation
SDMA	Symmetric dimethylarginine
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SI	Système international d'unités (International System of Units)
SSA	Sulfosalicylic acid
tCa^{2+}	Total calcium

Table 8.1. Abbreviations and symbols in this chapter (Continued)	
TS %	Total solids percentage (g/100 g)
UA	Urinalysis (urine analysis)
UN	Urea nitrogen
UN:Crt	Urea nitrogen to creatinine
UPC	Urine protein to creatinine
USG	Urine specific gravity
USG_{ref}	Refractometric urine specific gravity
WBC	White blood cell (leukocyte)

PHYSIOLOGIC PROCESSES

I. Three major processes control renal excretion of H_2O and solutes: (i) glomerular filtration (passive), (ii) tubular resorption (active or passive), and (iii) tubular secretion (active or passive).
   A. In a healthy person, roughly 25 % of the cardiac output enters the kidneys, which results in approximately 180 L of H_2O entering the renal tubules each day. About 99 % of that H_2O is resorbed by the tubules and thus only 1–2 L of urine is formed per day.[1] Specific information for other mammalian kidneys varies, but the key concept does not – nearly all of the H_2O that enters the tubules via glomeruli is resorbed by the tubules in the healthy mammal.
   B. One conceptual way of understanding the overall handling of solutes by the renal tubules is to compare the plasma and urine concentrations of various solutes to the plasma and urine Crt concentrations. In health, plasma [Crt] may be 0.1 mmol/L (100 µmol/L) while the urine [Crt] is 8 mmol/L, making the urine [Crt] 80 times the plasma [Crt]. As Crt is minimally secreted and not resorbed by the renal tubules, the urine [Crt] increases 80× because of H_2O resorption by the renal tubules.
   C. Table 8.2 provides illustrative concentrations of major urine solutes in comparison to Crt concentrations. Differences between plasma and urine [Crt] reflect the magnitude of H_2O resorption because Crt is minimally secreted and not resorbed by the renal tubules. Most solutes in the table do not get concentrated as much as Crt during their passage through the renal tubules because some of the solute is resorbed. However, some urine solutes are more concentrated (see NH_4^+) because the solute is added to the tubular fluid by the renal tubules.
   D. If assessment of renal tubular function related to a specific solute is needed, the rate of urinary excretion of a plasma substance is compared to the rate of inulin (not insulin!) excretion. If excretion of a solute is greater than excretion of inulin, there is a net solute secretion by tubules involving processes in which solutes from plasma, interstitial fluid, or tubular cells are transferred to tubular fluid. If excretion of a solute is less than excretion of inulin, there is a net solute resorption by tubules, or the substance does not freely pass through the GFB.

Table 8.2. Comparison of selected plasma and urine analyte concentrations in health[a]

Analyte	Plasma (mmol/L)	Urine[a] (mmol/L)	Renal effect on concentration
Crt	0.1	8	↑ 80× by H_2O resorption
Urea	7	210	Only ↑ 30× because some urea is resorbed
Glucose	5	0	All glucose is resorbed
Osmolality	300	900	Only ↑ 3× because most solutes are resorbed
Na^+	150	30	Decreased because more Na^+ than H_2O is resorbed
Cl^-	110	70	Decreased because more Cl^- than H_2O is resorbed
HCO_3^-	24	0	All HCO_3^- that enters tubular fluid is conserved
PO_4	2	100	Only ↑ 50× because PO_4 molecules are resorbed
NH_4^+	< 1	120	↑ > 100× because NH_4^+ is secreted by renal tubules

[a] Actual concentrations vary greatly depending on many factors and especially the renal conservation of H_2O; these concentrations are approximations.

E. Resorption of H_2O by tubules
   1. About 30 % of RPF becomes filtrate. About 75 % of the filtrate H_2O is passively resorbed in the PCTs.
   2. H_2O is passively resorbed in the descending limb of the LoH (because of an osmotic gradient) as the loop enters the hypertonic medulla.
   3. The ascending limb of the LoH and connecting segments are impermeable to H_2O.
   4. The collecting ducts are permeable to H_2O in the presence of ADH (Figure 8.1), which leads to insertion of AQP proteins into the cell membranes. H_2O moves through the cell membranes via pores at the center of each protein. Lack of ADH triggers endocytosis of AQP and its return to cytoplasmic vesicles.

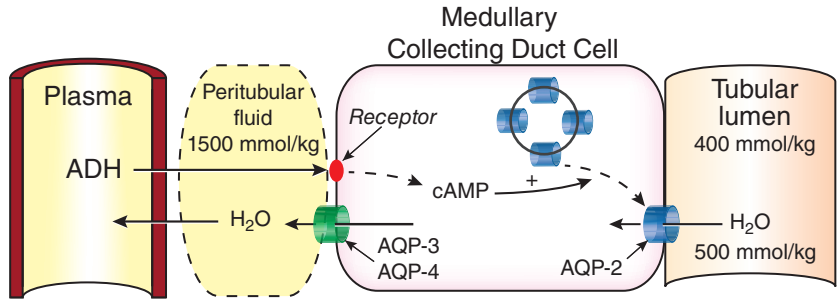


Figure 8.1. ADH actions. Either hypovolemia or hyperosmolal plasma stimulates the release of ADH from the pituitary gland. After binding to specific membrane receptors of DCT and collecting duct cells, activation of an adenylate cyclase system causes increased AQP-2 formation and insertion of AQP-2, some from recyclable storage vesicles, into the apical membrane. The AQP-2 acts as a pore for the passive movement of H_2O from the lumen into the cell. The peritubular membrane contains other aquaporin molecules (AQP-3, AQP-4) that allow H_2O to pass out of the cell via osmosis and into the plasma. The effect of H_2O resorption on the osmolality of tubular fluid is indicated by an increase in osmolality near the AQP-2 pore.

II. Glomerular filtration

A. A major route for solute and H_2O excretion from an animal is through renal glomeruli (Figure 8.2). The filtering function of glomeruli is typically assessed by evaluating the renal excretion of substances that pass freely through the GFB.

1. The potential for a substance to pass through the healthy GFB (from plasma to filtrate) depends on two major factors:
   a. Molecular size: Nearly 100 % of molecules with radius < 2.5 nm pass through. Almost no molecules with radius > 3.4 nm pass through.
   b. Electrical charge: Positively charged and electrically neutral substances pass through better than negatively charged substances because the glomerular basement membrane contains negatively charged molecules.
2. In many species, albumin ($M_r \cong 66{,}000–69{,}000$; 3.5 nm radius) is near the threshold size of the GFB; its negative charge also impedes transit. The small amount of albumin that crosses the GFB in health is mostly (> 90 %) resorbed by tubular epithelial cells; therefore, only very small amounts of albumin are in urine, although a small amount, sometimes detectable by routine tests, is commonly present in apparently healthy dogs. This albuminuria may represent subclinical disease or a species variation.

B. GFR

1. *GFR* is the rate at which fluid moves from plasma to glomerular filtrate, and is measured by determining the rate at which a substance is cleared from plasma.
2. GFR depends primarily on the rate of RPF and varies proportionately with RPF. RPF depends on blood volume, cardiac output, number of functional glomeruli, and

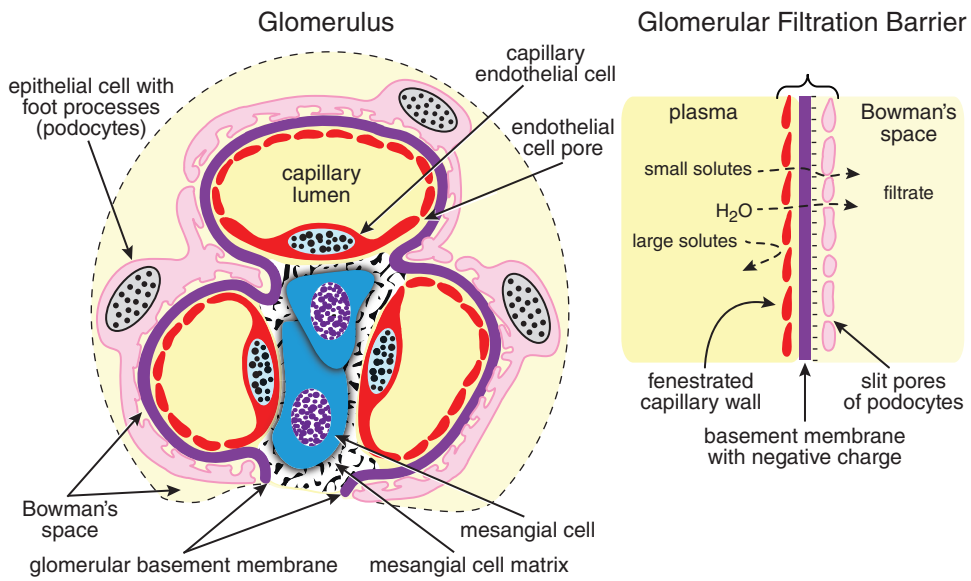


Figure 8.2. Glomerular filtration barrier. The GFB consists of the capillary endothelial cell, the glomerular basement membrane, and the epithelial cells (podocytes). H_2O and most solutes pass through fenestrations in the endothelial cells, through a semipermeable basement membrane, through the slit pores between the foot processes of the podocytes, into Bowman's space under and around the podocytes, and then into the PCT.

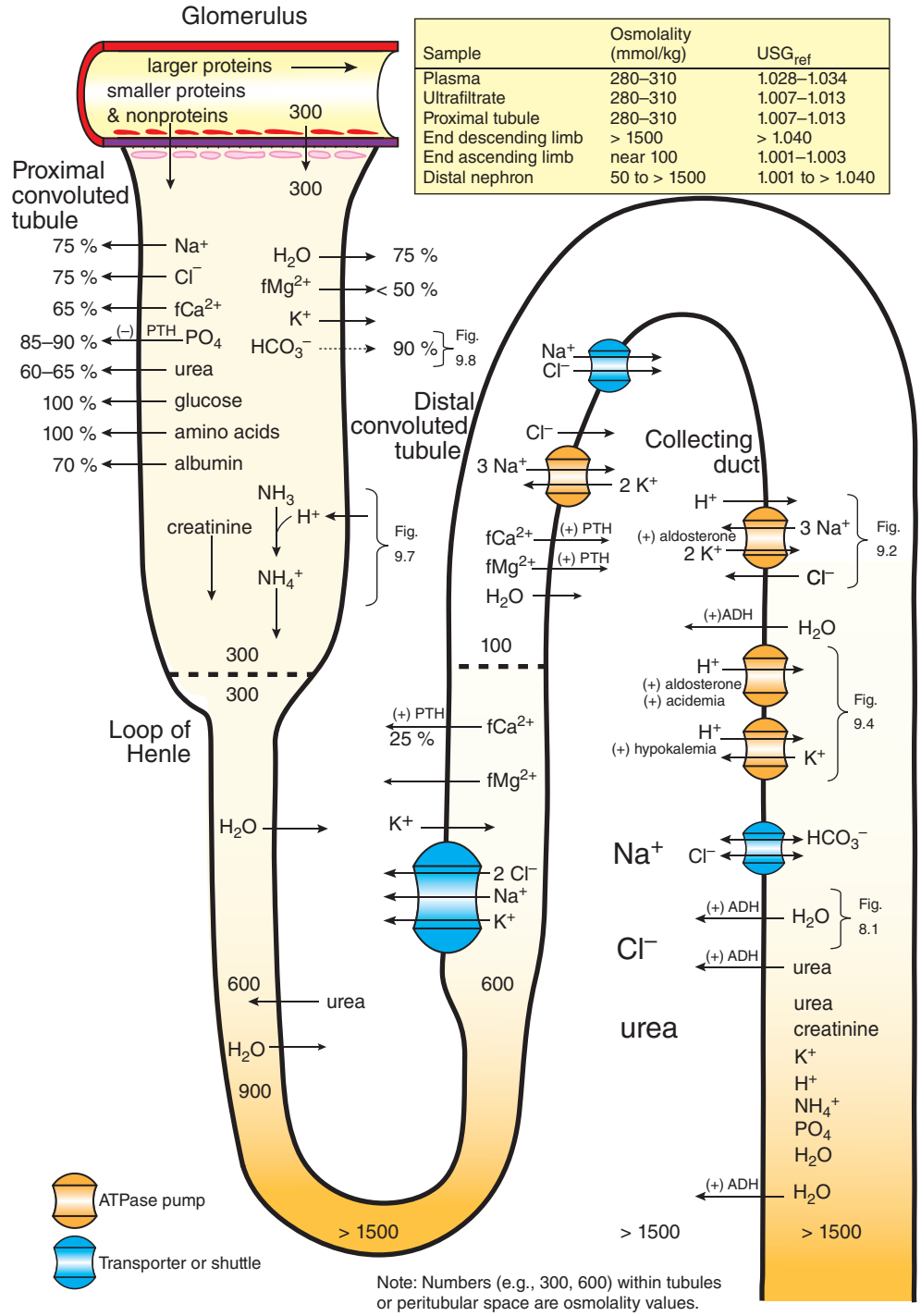


Figure 8.3. Major physiologic processes of renal tubules that pertain to solutes and H_2O. The solute concentrations are provided to illustrate changes that occur as the fluid moves through the nephron (Figure 8.4). Actual solute concentrations would vary, depending on many physiologic and pathologic factors.

- The osmolality of the plasma and the ultrafiltrate are equal (near 300 mmol/kg) as H_2O, nonprotein solutes, and smaller proteins pass through the GFB.

constriction or dilation of afferent and efferent glomerular arterioles. Other factors that affect GFR include intracapsular hydrostatic (hydraulic) pressure in Bowman's space (if pressure is increased, GFR is decreased) and plasma colloid osmotic (oncotic) pressure (if pressure is acutely increased, GFR is decreased).[2] Conversely, one would expect that hypoproteinemia (thus decreased oncotic pressure) would cause an increased GFR. However, hypoproteinemia induced by plasmapheresis over 2–3 wk caused decreased GFR in dogs because of a large decrease in glomerular hydrostatic pressure thought to be due to decreased mean arterial blood pressure and renal blood flow.[3] Similar findings (lower GFR, decreased renal blood flow) occur in people who have analbuminemia.[4]

3. The ideal solute for measuring GFR would not be protein bound, would pass freely through the GFB, and would be neither secreted nor resorbed by renal tubules. Inulin, iohexol, and mannitol nearly meet these criteria. Crt is often used because very little is secreted by renal tubules in most animals, and it meets the other criteria. GFR can also be assessed by imaging methods: excretion of radioactive compounds (renal scintigraphy) or contrast media (contrast nephrography).

III. Functions of tubules pertaining to major solutes[5] (Figure 8.3)

A. Na^+

1. About 75 % of filtrate Na^+ is resorbed in PCTs down a concentration gradient established by the Na^+-K^+-ATPase pump (basolateral membrane) and a Na^+-H^+ antiporter. Na^+ resorption is enhanced by an electrical gradient established by conservation of HCO_3^-; Na^+ is cotransported with glucose, amino acids, and

Figure 8.3. *continued*

- In the PCT, most of the H_2O and solutes that enter the tubules are resorbed through active, facilitated, and passive processes. The osmolality of the tubular fluid leaving the PCT is still near 300 mmol/kg, but the fluid volume is greatly diminished. In people, about 70 % of the small amount of filtrate albumin is resorbed in the PCT; about 90 % of the remaining albumin is resorbed in the LoH and distal nephron (not shown).
- In the descending limb of the LoH, tubular fluid is concentrated and volume reduced by the passive movement of H_2O. Urea may diffuse from the interstitial fluid to the tubular fluid. At the bottom of the LoH, the concentration of the tubular fluid varies among species. The 1500 mmol/kg value is probably appropriate for horses and cattle, whereas the solute concentration in cats may be > 2400 mmol/kg.
- In the ascending limb of the LoH, solutes (mostly Na^+, Cl^-, and K^+) passively leave the tubular fluid via a Na^+-K^+-$2Cl^-$ carrier (Na^+-K^+-ATPase pumps in the basolateral membrane create the gradient), but H_2O remains. Thus, the tubular fluid becomes dilute, and the fluid leaving the diluting segment has an osmolality near 100 mmol/kg. Passive fCa^{2+} and fMg^{2+} resorption depends on an electrical gradient promoted by the Na^+-K^+-$2Cl^-$ cotransporter and the recycling of K^+, whereas active fCa^{2+} and fMg^{2+} resorption is promoted by PTH.
- In the DCTs and collecting ducts, there are multiple processes involved in electrolyte balance, acid-base balance, and conservation of H_2O (see the text for specifics). The major actions of ADH are to promote resorption of H_2O and urea. The major actions of aldosterone are the resorption of Na^+ and Cl^- and the secretion of K^+ and H^+. The osmolality of the urine typically is > 600 mmol/kg and may be > 2000 mmol/kg.
- In most healthy domestic mammals, the net function of a nephron is to excrete urea, Crt, K^+, H^+, NH_4^+, and PO_4 and to conserve Na^+, Cl^-, HCO_3^-, fCa^{2+}, fMg^{2+}, glucose, amino acids, and H_2O. The equine nephron excretes fCa^{2+} instead of conserving it.

The table in the top of the figure shows a comparison of the approximate solute concentrations (in terms of osmolality and USG_{ref}) in different segments of a functional nephron. Note that the "USG_{ref}" interval for plasma (1.028–1.034) was determined by using a refractometer's USG scale. The true specific gravity of the plasma would be nearer 1.018–1.022;[6] these intervals are different from the isosthenuric USG_{ref} interval of 1.007–1.013 that occurs when urine and plasma osmolalities are nearly equal.

phosphates. Angiotensin II stimulates the proximal tubular resorption of Na^+, Cl^-, and H_2O. Atrial natriuretic peptide inhibits the Na^+-K^+-ATPase pump and thus increases Na^+ excretion.[7]

2. Na^+ is passively resorbed into the descending limb of the LoH to maintain Na^+ in the *countercurrent system*, the mechanism for maintaining medullary hypertonicity through the coordinated functions of juxtaposed descending and ascending limbs of the LoH along with the adjacent interstitium and surrounding blood capillary network (vasa recta renis).
3. Na^+ accompanies K^+ and Cl^- that are resorbed in the thick ascending limb of the LoH via a Na^+-K^+-$2Cl^-$ cotransporter in the luminal membrane. The rate-limiting factor is Cl^- delivery to the loop. Furosemide diuretics block this process. Atrial natriuretic peptide inhibits the Na^+-K^+-$2Cl^-$ cotransporter and thus increases Na^+ excretion.[7]
4. ADH stimulates Na^+ and Cl^- resorption in the medullary thick limb of the LoH through the Na^+-K^+-$2Cl^-$ cotransporter (a minor role for ADH).
5. Aldosterone stimulates the active resorption of Na^+ in collecting ducts by opening Na^+ channels, enhancing Na^+-K^+-ATPase activity in the basolateral membrane, and opening luminal K^+ channels. Its actions are probably mediated through aldosterone-induced proteins (Na^+-K^+-ATPase may be one of the proteins) (Figure 9.2).
6. Na^+ and Cl^- resorption in the DCT also involves an aldosterone-independent Na^+-Cl^- cotransporter. This process varies directly with Na^+ delivery to the distal nephron. Thiazide diuretics block this cotransporter.

B. Cl^-

1. About 75 % of filtered Cl^- is resorbed in the PCTs down a concentration gradient created by Na^+ and H_2O resorption and through a formate-Cl^- exchanger (formic acid is an intermediate in one-carbon metabolism).
2. Cl^- is resorbed via a Na^+-K^+-$2Cl^-$ cotransporter in the thick ascending limb of the LoH.
3. Cl^- is passively resorbed in the distal nephron by an electrochemical gradient established by Na^+ movement through aldosterone and aldosterone-independent processes (Figure 9.2).
4. Cl^- is also secreted by the type A intercalated cells when they are stimulated to secrete H^+. Concurrent with the secretion of Cl^-, HCO_3^- is produced by the cells and enters the plasma (Figure 9.4).

C. HCO_3^-

1. About 90 % of filtered HCO_3^- is conserved indirectly in the PCTs during H^+ secretion. H^+ secretion is mediated by the Na^+-H^+ antiporter and depends on Na^+ resorption. As Na^+ is resorbed, the secreted H^+ combines with HCO_3^- in the filtrate to form H_2CO_3, which then forms CO_2 and H_2O. The CO_2 and H_2O enter the PCT cells in which they are converted to H^+ (which is again available for secretion) and HCO_3^-. The HCO_3^- is transported to the peritubular fluid via a Na^+-$3HCO_3^-$ symporter (Figure 9.8).
2. In the collecting ducts, HCO_3^- produced by the type A intercalated cells enters the peritubular fluid through a Cl^--HCO_3^- exchanger (Figure 9.4). A reverse process in type B intercalated cells leads to HCO_3^- secretion when there is excess HCO_3^- (Figure 9.9).

D. K^+

1. In health, most K^+ that enters the PCT through the GFB is resorbed prior to the DCT. K^+ is secreted primarily by the principal cells of the collecting ducts, and secretion is promoted by aldosterone (Figure 9.2). K^+ movement from cell to tubular lumen occurs through K^+ channels opened by aldosterone. The movement is enhanced when the

urinary flow rate through the tubule is high. With high flow rates, the K^+ is washed away quickly, and thus a concentration gradient is maintained. The movement is reduced when the urinary flow rate through the tubule is low because K^+ stays longer in the tubular fluid, and thus the concentration gradient is diminished.

2. ADH promotes K^+ secretion in the cortical collecting ducts, perhaps by opening K^+ channels in the luminal membrane. This process compensates for the reduced K^+ secretion that occurs with decreased urinary flow.
3. Type A intercalated cells conserve K^+ when hypokalemia is present (Figure 9.4).

E. H^+

1. H^+ is secreted from type A intercalated cells of the cortical collecting ducts through a H^+-ATPase pump that can work against a large concentration gradient (Figure 9.4). Aldosterone and acidemia promote this process. H^+ is also secreted and K^+ is resorbed through H^+-K^+-ATPase pumps that appear to be most active when hypokalemia is present.
2. A limited amount of H^+ is secreted by the PCT cells (Figure 9.8), after which it may be buffered by HCO_3^-, NH_3, or PO_4. Most of the renal excretion of H^+ is within NH_4^+ (Figure 9.7) and either HPO_4^{2-} or $H_2PO_4^-$. Very little is present as free H^+ (~10^{-8}–10^{-6} mol/L).

F. Ca^{2+}

1. In most mammals, nearly 90 % of filtrate Ca^{2+} is resorbed by renal tubules (see Chapter 11, Total and Free Calcium (tCa^{2+}, fCa^{2+}) Concentrations I.B.5). In horses, however, renal excretion of Ca^{2+} is a major mechanism of eliminating excess dietary Ca^{2+} (see Chapter 11, Total and Free Calcium (tCa^{2+}, fCa^{2+}) Concentrations V.B.1.a).
2. PTH promotes Ca^{2+} resorption in the cortical thick ascending limb of the LoH, the DCT, and the connecting segment between the DCT and the collecting ducts (see Chapter 11, Total and Free Calcium (tCa^{2+}, fCa^{2+}) Concentrations I.B.5).
3. Vitamin D promotes Ca^{2+} resorption in the distal nephron by inducing production of a calcium-binding protein. In most mammals, $1,25(OH)_2D$ formation in the PCTs is stimulated by increased PTH activity and hypophosphatemia. A major species variation is that equine kidneys lack the enzyme needed to form $1,25(OH)_2D$ (see Chapter 11, Vitamin D Concentration I.A.2.b).
4. Thiazide diuretics increase the resorption of Ca^{2+} in the distal nephron.

G. PO_4

1. About 85–90 % of filtrate PO_4 is resorbed in the PCT via the actions of a Na^+-PO_4 cotransporter.
2. The cotransporter's activity is enhanced by hypophosphatemia and insulin, whereas it is diminished by hyperphosphatemia, FGF23, and increased PTH activity (see Chapter 11, Inorganic Phosphorus (Pi) Concentration, I.D.1).

H. Mg^{2+}

1. At physiologic concentrations, nearly all filtrate Mg^{2+} is resorbed (see Chapter 11, Total and Free Magnesium (tMg^{2+}, fMg^{2+}) Concentrations, I.C.3).
2. ADH, PTH, glucagon, calcitonin, and β-adrenergic agonists stimulate Mg^{2+} resorption in the cortical thick ascending limb.

I. Glucose

1. Typically, glucose passes freely through the GFB, and all filtrate glucose is resorbed in the PCTs as Na^+ is resorbed by a Na^+-glucose cotransport system involving glucose transporter proteins. The glucose resorption process is called *secondary active transport* because it occurs *secondary* to Na^+ resorption and because it is *active*, i.e., it requires

energy to move up a concentration gradient (there is a higher concentration in cells than in tubular fluid in the healthy state).

2. The transport process involves carrier proteins that can be saturated by excessive glucose from plasma, thus producing hyperglycemic glucosuria.

J. Proteins and amino acids

1. Nearly all filtrate proteins and amino acids are resorbed in the PCTs.
2. Amino acids are resorbed through several amino acid transporters with distinct affinities for different types of amino acids (e.g., neutral, cationic, aromatic). Small peptides are hydrolyzed at the brush border, and the amino acids are resorbed.
3. Larger proteins (including albumin), if they reach the tubular lumen, enter the tubular cells through receptor-mediated endocytosis and then are degraded to amino acids. Megalin, amnionless, and cubilin are receptor proteins expressed in the apical part of PCT cells and are important in mediating endocytosis of a wide variety of proteins. Tubular absorption of albumin is dependent on cubilin, and megalin promotes the internalization of cubilin/albumin complexes.[8] Vitamin D–binding protein, which is required for renal formation of $1,25(OH)_2D$, also is absorbed via these receptor proteins. Cubilin also serves as the receptor protein for the intrinsic factor–cobalamin complex in the small intestine (see Chapter 15, Figure 15.2).
4. Little to no albumin is detectable in the urine of most healthy individuals, but mild proteinuria is detected by routine UA of samples from some apparently healthy dogs. This urinary protein may represent subclinical disease or a species difference.

K. Urea

1. About 60–65 % of filtrate urea is resorbed in the PCTs down a concentration gradient created by the movement of H_2O into the cells, which initially lowers cellular [urea] and raises tubular [urea]. This process is enhanced in hypovolemic states when there is greater proximal tubular resorption of Na^+ and H_2O, decreased urine flow rate, and thus more time for passive urea absorption.
2. Urea is recycled in the remaining nephron by moving from interstitial fluid to fluid in the LoH and then from medullary collecting duct fluid to medullary interstitial fluid.
3. Urea resorption in the medullary collecting ducts is enhanced by ADH activity and a concentration gradient established by H_2O resorption secondary to Na^+ and Cl^- resorption. ADH increases tubular permeability to urea by enhancing the activity of urea-transporters including urea-transporter A1 protein in the apical plasma membranes of medullary collecting duct epithelium.[9] Urea transporters are needed to facilitate movement of urea, a molecule that is polar and therefore otherwise diffuses relatively slowly through cell membranes.
4. In health, about 50 % of the filtrate urea is resorbed by the combined actions of the renal tubules. Urea contributes nearly 50 % of interstitial solute for the establishment of a hypertonic medulla that is necessary for renal concentrating ability.

L. Crt

1. In some dogs, small amounts of Crt are secreted by the PCTs.[10,11] Crt secretion does not appear to occur in horses or cats.[12,13]
2. In people, Crt secretion occurs via organic cation transporters, organic anion transporters, and multidrug and toxin extrusion proteins. Mild but potentially clinically significant increases in plasma [Crt] can occur with impairment of this secretion caused by drugs including cimetidine, trimethoprim, quinidine, and tyrosine kinase inhibitors.

IV. Renal concentrating ability and renal diluting ability
  A. Definitions
    1. *Concentrating ability*: the ability of kidneys to resorb filtrate H_2O in excess of filtrate solutes; ability to concentrate solutes
    2. *Diluting ability*: the ability of kidneys to resorb filtrate solute in excess of filtrate H_2O; ability to dilute solutes
  B. Terms used to describe the concentration of urine
    1. Authors may describe the solute concentration of urine with the terms hyposthenuria, isosthenuria, and hypersthenuria (*-sthen-* means "strength"). However, there is little agreement on the definitions for these terms. Based on its roots, isosthenuria means same (*iso-*) strength (*-sthen-*) urine (*-uria*).
    2. Calling a urine "dilute" or "concentrated" can lead to confusion.
      a. If a urine's osmolality is less than the osmolality of the glomerular filtrate from which it formed, then the kidneys have diluted the filtrate; the urine is more dilute than the filtrate. Such urine is typically considered dilute.
      b. If a urine's osmolality is greater than its corresponding glomerular filtrate osmolality, then the kidneys have concentrated the filtrate; the urine is more concentrated than the filtrate. Such urine is concentrated to at least some extent, but it may not be adequately concentrated for an animal's hydration status.
      c. If one urine sample has a USG_{ref} of 1.050 and a second urine sample has a USG_{ref} of 1.025, the second is dilute compared to the first, but both are concentrated compared to the glomerular filtrate, which has a USG_{ref} near 1.010 in health.
    3. Our definitions (see Figure 8.11 for relationship of USG_{ref} and urine osmolality)
      a. *Isosthenuria* (working definition) is the state in which urine osmolality is the same as plasma osmolality, whether plasma osmolality is decreased, within RI, or increased. We define "the same" as being within 100 mmol/kg of plasma osmolality; that is, $osmolality_u = osmolality_p \pm 100$ mmol/kg. In most domestic mammals, such urine typically has a USG_{ref} of 1.007–1.013 (authors' unpublished data). As shown in Figure 8.11, if the measured $osmolality_u$ is 300 mmol/kg, the observed USG_{ref} is 1.007–1.013. This is similar to the often-used 1.008–1.012 interval. Considering the differences in refractometer calibrations, it is difficult to establish one isosthenuric interval for refractometric specific gravity values. Also, the interval will be different if there is plasma hyperosmolality or hypoosmolality.
      b. *Hyposthenuria* is the state in which excreted urine has an osmolality that is less than the isosthenuric values; that is, $osmolality_u < (osmolality_p - 100$ mmol/kg). In most hyposthenuric animals, the USG_{ref} is < 1.007 but it may be mildly higher or lower with plasma hyperosmolality or hypoosmolality, respectively. Such urine is dilute (the filtrate has been diluted by renal processes).
      c. *Eusthenuria* (an uncommonly used term) is the excretion of urine with the osmolality expected for an animal that has adequate renal function and normal hydration status, e.g., a canine urine with an osmolality of 900 mmol/kg (approximately a USG_{ref} of 1.030). The USG_{ref} values in Table 8.3 could be called eusthenuric values.
      d. *Hypersthenuria* (an uncommonly used term) is the excretion of highly concentrated urine. USG_{ref} values above those in Table 8.3 could be called hypersthenuric values.
      e. USG_{ref} values above the isosthenuric interval indicate that the renal tubules have concentrated the glomerular filtrate if the USG_{ref} is accurately reflecting osmolality and there is not hyperosmolal plasma.

Table 8.3. Expected UA results in healthy dogs, cats, horses, cattle, and camelids

	Dogs	Cats	Horses	Cattle	Camelids[14]
Physical					
Color	Yellow[a]	Yellow[a]	Yellow[a]	Yellow[a]	Yellow[a]
Clarity	Clear	Clear	Hazy-turbid	Clear	Clear
USG_{ref}	1.015–1.050[b]	1.035–1.060[b]	1.020–1.050[b]	1.020–1.045[b]	1.018–1.050
Chemical					
pH	5.5–7.5	5.5–7.5	7.5–8.5	7.0–8.5	7.0–8.5
Protein	Neg–1+[c]	Neg	Neg	Neg[d]	Neg
Glucose	Neg	Neg	Neg	Neg	Neg
Ketone	Neg	Neg	Neg	Neg[e]	Neg
Heme	Neg	Neg	Neg	Neg	Neg
Bilirubin	Neg–1+[c]	Neg	Neg	Neg	Neg
Urobilinogen (EU)	0.2–1.0	0.2–1.0	0.2–1.0	0.2–1.0	0.2–1.0
Sediment (from centrifugation of 5 mL of fresh urine)					
WBCs/hpf	< 5[f]	< 5[f]	< 5[f]	< 5[f]	< 5[f]
RBCs/hpf	< 5	< 5	< 5	< 5	< 5
Bacteria/hpf	None	None	None	None	None
Casts/lpf	Few[g]	Few[g]	Few[g]	Few[g]	Few[g]
Epithelial cells/lpf	None to few[h]	None to few[h]	None to few[h]	None to few[h]	None to few[h]
Crystals/lpf	Varies[i]	Varies[i]	Varies[i]	Varies[i]	Rare
Other	—	—	Mucus	—	—

[a] The intensity of yellow usually varies proportionately with the USG_{ref}.
[b] Assuming normal hydration status and no treatments that alter H_2O resorption by the kidneys
[c] Trace and 1+ reactions occur in the more concentrated samples.
[d] Many 1+ and 2+ reactions were seen in catheterized samples using Combur-Test strips.[15]
[e] Although mild reactions occur in high-producing dairy cattle without apparent disease, they reflect a caloric imbalance.
[f] The number of cells seen per microscopic field varies with the method (see text) and with the diameter of the viewed field, which depends on microscope ocular lenses.
[g] A few hyaline casts usually are not associated with a pathologic state. Occasional granular casts can be found in healthy animals.
[h] This varies with the method of collection. Large round epithelial cells (transitional or squamous) and angular cells (squamous) are expected in voided and some catheterized samples.
[i] Phosphate crystals in dogs and cats and carbonate crystals in horses and cattle are common without disease, and other crystals may be present as well (Table 8.15); crystals not expected in health include cystine, tyrosine, leucine, xanthine, bilirubin in cats, and usually calcium oxalate monohydrate (except hemp seed variant in horses) and ammonium biurate.

C. Physiologic processes for concentrating or diluting glomerular filtrate
   1. Functions of renal interstitium
      a. Provides a bridge between tubules and blood vessels to facilitate the movement of H_2O and solutes
      b. Helps maintain medullary hypertonicity to enable the concentration of tubular fluid

2. Functions of tubules (Figure 8.4)
   a. PCT
      (1) Approximately 30 % of the plasma that enters glomeruli becomes glomerular filtrate ($USG_{ref} \cong 1.010$; osmolality ≅ 300 mmol/kg).
      (2) About 75 % of the H_2O in the filtrate is passively resorbed by the PCTs (independent of body needs). The remainder passes into the LoH. The solute concentration does not change much in the PCT, but fluid volume decreases markedly.
   b. Descending limb of the LoH
      (1) The osmolality of tubular fluid increases because of the passive resorption of H_2O and the secretion of Na^+, Cl^-, and urea into the filtrate.
      (2) Fluid entering the LoH has a USG_{ref} of ~1.010 and an osmolality of ~300 mmol/kg. Tubular fluid at the distal end of the descending limb is highly concentrated ($USG_{ref} > 1.050$; osmolality > 1500 mmol/kg).

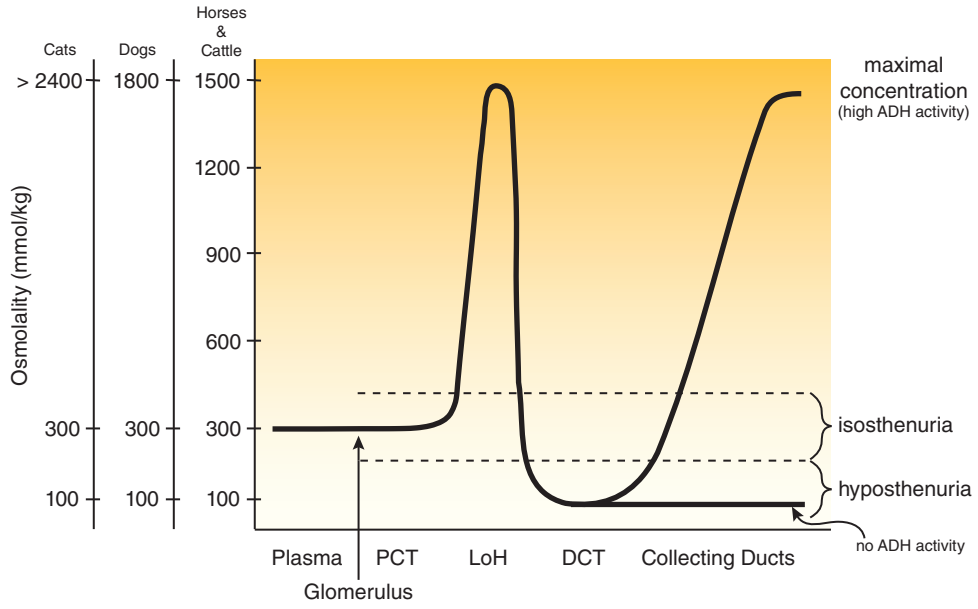


Figure 8.4. Osmolality changes in a healthy mammal's nephron. (Details of the movement of solute and H_2O in nephrons are described in Figure 8.3.) The ultrafiltrate formed by glomerular filtration of plasma has an osmolality near 300 mmol/kg. As fluid moves through the PCT, most of the H_2O and solutes are resorbed. Because resorption is isoosmotic, the tubular fluid's osmolality does not change. However, there are marked changes in osmolality during fluid transit through the LoH. When fluid leaves the LoH, the tubular fluid's osmolality is near 100 mmol/kg. Minimal change in osmolality occurs in the DCT as relatively few electrolytes are resorbed. In the absence of ADH activity, H_2O remains in the collecting ducts, and thus the nephron produces hyposthenuric urine (typically $USG_{ref} < 1.007$). With minimal ADH activity in healthy mammals, the nephron produces isosthenuric urine (typically USG_{ref} of 1.007–1.013). With marked ADH activity in the collecting ducts, a large portion of the H_2O is resorbed to form maximal concentration. The maximal concentrating ability varies among domestic mammals. The right *y*-axis is probably appropriate for horses and cattle (i.e., maximum $USG_{ref} \cong 1.050$), whereas other *y*-axes are needed for dogs (≅ 1800 mmol/kg or $USG_{ref} \cong 1.060$) and cats (> 2400 mmol/kg or $USG_{ref} > 1.080$). The yellow colors of urine depend on urochrome concentrations; typically, pale yellow or colorless urine is more dilute and dark yellow urine is more concentrated.

c. Ascending limb of the LoH (the diluting segment of the nephron)
   (1) The ascending limb is relatively impermeable to H_2O but actively pumps Cl^- and Na^+ (also K^+, fCa^{2+}, and fMg^{2+}) from the tubular fluid to the interstitial fluid. Thus, tubular fluid loses solute, and the interstitial fluid becomes more hypertonic. Fluid leaving the ascending limb for the DCT is dilute (USG_{ref} < 1.007; osmolality < 200 mmol/kg).
   (2) A functional ascending limb is necessary to maintain a hypertonic interstitial fluid in the medullary region so that H_2O may be passively resorbed in the descending limb of the LoH and in collecting ducts.
d. DCT
   (1) The DCT has minimal H_2O permeability, and thus very little H_2O is resorbed in this segment.
   (2) The resorption of Na^+ and Cl^- in this segment lowers the tubular fluid osmolality.
e. Collecting ducts
   (1) The collecting ducts contain the concentrating processes of the nephron.
   (2) ADH controls permeability of the epithelium to H_2O. H_2O is resorbed in the presence of ADH if there is an osmolar gradient between the tubular fluid and medullary interstitial fluid. An osmolar gradient is created by high concentrations of urea, Na^+, and Cl^- in the interstitial fluid that are produced and maintained by segmental functions of the nephron. The H_2O channels through the epithelial cells are created by membrane-associated proteins AQPs (Figure 8.1).

3. For kidneys to concentrate the filtrate, the following are necessary:
   a. ADH must be present. Stimuli for ADH secretion include hyperosmolality, decreased cardiovascular pressures as seen with hypovolemia, and, to a lesser degree, increased [angiotensin].
   b. Epithelial cells of the collecting ducts must be responsive to ADH.
   c. There must be a concentration gradient; that is, the osmolality of the interstitial fluid of the renal medulla must be greater than the osmolality of the fluid in the tubules.
4. For kidneys to dilute the filtrate, the following are necessary:
   a. Na^+ and Cl^- must be actively transported from the tubular fluid to the interstitial fluid by epithelial cells of the ascending limb of the LoH. This process requires delivery of Na^+ and Cl^- to the LoH.
   b. Very little to no H_2O is removed from the tubular fluid by the collecting ducts.

CHRONIC RENAL DISEASE, INSUFFICIENCY, OR FAILURE

I. What are chronic renal disease, insufficiency, and failure?
   A. Generically, chronic renal (kidney) disease is any persistent (months, years) disorder involving one or both kidneys, from a benign renal cyst requiring no intervention to end-stage renal failure. However, *chronic renal disease* typically refers to disorders lasting at least 90 d that are characterized by progressive loss of renal function with associated decreases in GFR. Many chronic diseases can damage kidneys enough that functional renal tissue is inadequate to maintain health. Then, the animal enters the pathophysiologic state of chronic renal insufficiency or failure. Many veterinarians do not differentiate chronic renal

insufficiency from chronic renal failure as they share two criteria: (i) evidence of decreased GFR (typically azotemia) and (ii) evidence of impaired concentrating ability (unable to adequately concentrate solutes when challenged by dehydration).

B. The following longstanding system for staging impaired renal function is conceptually useful and correlates with clinical findings seen in many domestic mammals with progressive renal disease.[16,17]
   1. Diminished renal reserve: The GFR is about 50 % of normal, and the animal is clinically healthy. Azotemia is not present, but the kidneys are less able to tolerate additional insult (e.g., disease, dehydration, or poor perfusion).
   2. Chronic renal insufficiency: The GFR is approximately 20–50 % of normal. Azotemia and anemia appear. Polyuria occurs because of decreased concentrating ability.
   3. Chronic renal failure: The GFR is < 20–25 % of normal. Azotemia and impaired concentrating ability (thus polyuria) are present. The kidneys cannot regulate extracellular fluid volume or electrolyte balance, and thus edema, hypocalcemia (generally not in horses), and metabolic acidosis develop. Overt uremia with its neurologic, gastrointestinal, and cardiovascular complications may develop.
   4. End-stage renal disease: The GFR is < 5 % of normal. The terminal stages of uremia are present with either oliguria or anuria.
   5. Figure 8.5 illustrates the development of azotemia and abnormal urine volume caused by a progressive loss of nephrons.

C. This staging system fits the concept that impaired renal function develops progressively with a decreasing number of functional nephrons (which may result from primary glomerular, tubular, or interstitial diseases), thus decreasing functional renal mass.
   1. The basic concept of the "intact-nephron hypothesis" is that a nephron is a functional unit. If there is sufficient glomerular damage that the glomerulus is not functional (does not filter plasma), then the remainder of that nephron dies and thus there is reduced renal functional mass. If a tubular segment of the nephron is irreversibly damaged and no longer allows the flow of fluid, then the remainder of that nephron dies.
   2. However, selectively impaired renal function may occur when certain portions of the nephron are dysfunctional (e.g., defective glomeruli causing proteinuria or defective solute resorption by PCTs) without enough loss of functional nephrons to impair clearance of metabolic waste or conservation of H_2O. There have been attempts to identify biomarkers of renal injury that may or may not progress sufficiently to decrease functional renal mass.

D. It is commonly stated that more than two-thirds of functional renal mass must be lost before kidneys lose the ability to concentrate urine (i.e., they produce isosthenuric urine), and more than three-fourths must be lost before an animal becomes azotemic. Such fractions are useful guidelines but do not represent firmly established facts for all species. In one study in which renal function was reduced by nephrectomy and selective arterial ligation, three of three cats with an estimated 83 % loss of functional renal mass became azotemic and maintained renal concentrating ability (USG_{ref} > 1.040) when tested during the 1–13 wk following surgery.[18]

II. Why do animals lose renal concentrating ability in chronic renal failure?
   A. More solute than usual is presented to the remaining functional nephrons, and the high solute content within the tubules contributes to solute diuresis.
   B. Medullary hypertonicity is not maintained because of three factors:
      1. Medullary tissue is damaged or medullary blood flow is abnormal.

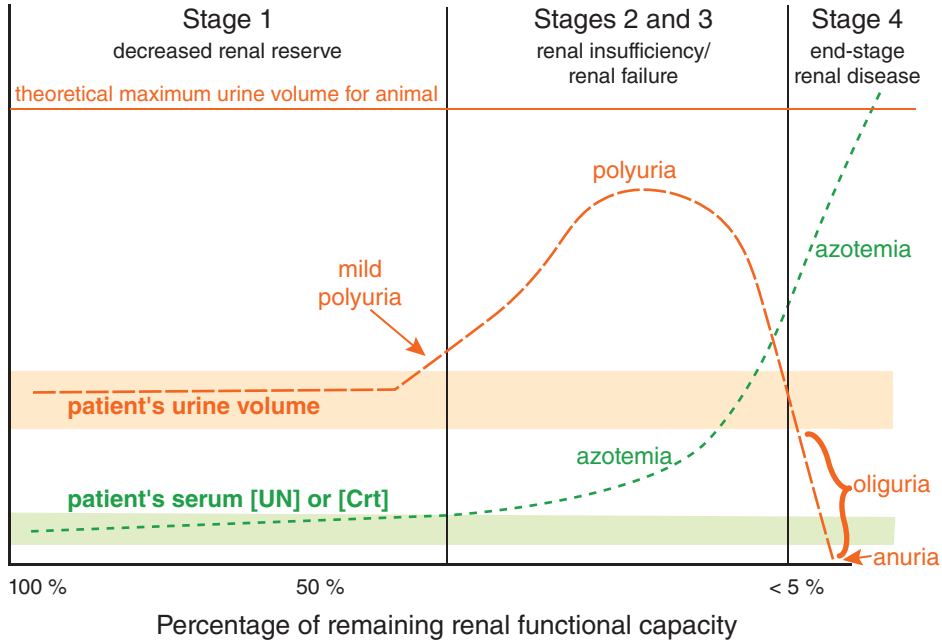


Figure 8.5. Graphical representation of the effects of chronic renal disease and progressive dysfunction on an animal's urine volume and serum concentrations of UN or Crt. *Shaded areas* represent the RIs for urine volume (*pale orange*) and for [UN] or [Crt] (*pale green*). The patient's urine volume and [UN] or [Crt] are represented by *labeled dashed lines*. A theoretical *maximal urine volume line* is shown to illustrate that animals with polyuric renal failure do not produce maximal urine volume.

- In *stage 1* (diminished renal reserve), progressive renal disease is destroying nephrons and thus GFR decreases. However, there is still sufficient function to clear most urea and Crt, and thus the animal is not azotemic using population-based RIs. Also, there is sufficient ability to concentrate urine, and thus polyuria is absent or not detected. As the renal dysfunction approaches stage 2, mild polyuria may develop because of impaired concentrating ability.
- In *stage 2* (renal insufficiency), enough nephrons have been lost for the renal concentrating ability to decrease (isosthenuria and polyuria develop); the excretion of urea and Crt by the kidneys is insufficient (azotemia is present).
- In *stage 3* (renal failure), there is continued isosthenuria, polyuria, and azotemia but also inadequate control of H_2O balance or electrolyte concentrations. The animal has clinical signs of uremia and abnormal serum concentrations of Na^+, K^+, Cl^-, Ca^{2+}, PO_4, H^+, or HCO_3^-.
- In *stage 4* (end-stage renal disease, oliguric or anuric renal failure), few nephrons are filtering plasma, and thus marked azotemia develops. The animal also has become oliguric or anuric because very little plasma H_2O enters the kidney. The remaining tubules cannot concentrate or dilute the filtrate, and thus USG_{ref} reflects isosthenuria.

2. Na^+ and Cl^- transport is decreased from the ascending limb of the LoH and thus Na^+ and Cl^- concentrations decrease in the interstitial fluid.
3. Damaged cells in the collecting ducts are less responsive to ADH and thus there is a reduced ability to conserve urea, a major contributor to hyperosmolal interstitial fluid in the renal medulla.

III. Polyuria in mammals with chronic renal insufficiency or failure
  A. The degree of polyuria in renal failure is not as severe as with other diuretic states, such as diabetes insipidus, because a decrease in functional nephrons decreases the volume of filtered plasma H_2O. The urine volume produced by healthy people is about 1–2 L/d, about 3–4 L/d with polyuric renal failure, and about 6–8 L/d with other polyuric states (e.g., central and renal diabetes insipidus).
  B. Mammals with chronic renal disease typically lose concentrating ability (and thus have polyuria) before azotemia develops. Cats can concentrate glomerular filtrate to a greater degree than other domestic species and may, when azotemic, retain more concentrating ability than other species.
  C. Progression of renal disease and loss of additional nephrons eventually causes either oliguric or anuric renal failure.

IV. Evidence of chronic renal insufficiency or failure
  A. Evidence of insufficiency or failure
    1. There is azotemia (increased [UN] and/or [Crt] in serum or plasma) because of inadequate renal ability to remove metabolic wastes from plasma.
    2. There is an inappropriately low USG_{ref} (often 1.007–1.013, but no lower) because of a marked reduction in renal concentrating (and diluting) ability.
  B. Evidence of chronicity
    1. Clinical findings, including duration of signs
    2. Laboratory findings, including anemia and, except in horses, hypocalcemia (although hypocalcemia may also occur in acute renal failure, and chronic renal failure may be associated with hypercalcemia, especially in horses)

ACUTE RENAL INJURY, IMPAIRMENT, OR FAILURE

I. Terms
  A. *Acute renal failure* can be considered reversible or irreversible renal dysfunction resulting abruptly (within hours to days) from a renal insult that markedly decreases GFR and leads to azotemia. In other words, the kidneys acutely fail to clear metabolic waste, but the failure may be transient. Usual causes are toxicants, renal ischemia, or infections. Patients may recover completely or develop chronic renal disease. Mild renal dysfunction can be considered *renal impairment* rather than failure.
  B. In human medicine, because of inconsistent criteria for acute renal failure and advances in diagnostics for its early detection, the term *acute kidney injury* was adopted to replace *acute renal failure* and describe a spectrum of acute renal disorders from mild changes in function to those requiring renal replacement therapy (i.e., from subclinical injury to acute renal failure).[19] Veterinarians now commonly refer to acute renal failure as acute kidney injury, but that label alone does not differentiate subclinical injury from clinical failure of the kidneys to clear metabolic waste and concentrate urine.
  C. Criteria for recognizing and staging human acute kidney injury (acute renal injury) continue to evolve but are based largely on increases in [Crt] and decreases in urine output. A similar approach has been adopted in veterinary medicine. Grading criteria for acute kidney injury in cats and dogs have been proposed (www.iris-kidney.com).
  D. Acute renal disease may occur in an animal with prior chronic renal disease. This is called *acute-on-chronic* disease.

II. Azotemia
   A. The magnitude of the azotemia does not differentiate acute from chronic renal failure. Both can have mild to severe azotemias.
   B. The rates of increase of the [urea] and [Crt] are greater in acute renal failure than in chronic renal failure; a moderate to marked azotemia may develop within days in acute failure but take weeks to months to develop with chronic failure.

III. Urine volume and USG_{ref}
   A. Because of the abrupt and severe decrease in GFR, the kidneys may filter little blood and produce little or no urine (oliguria or anuria).
   B. The USG_{ref} can vary considerably.
      1. The urine may be unexpectedly concentrated if it was formed prior to the severe insult.
      2. The urine may be isosthenuric because of the same mechanisms that impair concentrating ability in chronic renal disease.
      3. The urine is not expected to be hyposthenuric because such a state would indicate loss of concentrating ability but retained diluting ability.

IV. Acid-base status and electrolyte concentrations can become acutely abnormal (e.g., hyperkalemia or acidemia).

STAGING OF CHRONIC RENAL DISEASE IN DOGS AND CATS BY THE INTERNATIONAL RENAL INTEREST SOCIETY (IRIS)

I. IRIS is an organization that is dedicated to improving the understanding and treatment of renal disease in dogs and cats. Its website (www.iris-kidney.com) contains guidelines for the identification and staging of chronic renal disease. Table 8.4 contains the major aspects of the staging that is based largely on repeated Crt and SDMA concentrations from fasted, stable, hydrated patients. It is important to recognize that animals may have a chronic renal disease that has not reduced the number of functional nephrons sufficiently to create a state of renal insufficiency or failure. Further substaging is based on blood pressure and proteinuria (nonproteinuric, borderline proteinuric, or proteinuric).

II. The Crt and SDMA concentrations in Table 8.4 should not be used as firmly established decision limits because of biological variations and the analytical variation that is present within and among Crt and SDMA assays.[20] Table 8.5 includes data extracted from proficiency testing results of the Veterinary Laboratory Association. Aliquots of pooled sera were distributed to subscribing laboratories four times per year and the results of various methods of measuring Crt concentrations were used to generate the data. Extracted data include concentrations near the decision limits of the IRIS stages, where interlaboratory variation would affect classification. The data indicate that different laboratories obtain sometimes substantially different serum Crt concentrations when they analyze aliquots from one serum pool, and thus one set of decision limits is not appropriate for Crt assays of different laboratories. Potential reasons for the differences include assay calibration, inherent analytical imprecision, and different analytical methods (see Analytical concepts for Creatinine (Crt) Concentration in Serum or Plasma).

Table 8.4. IRIS staging of chronic renal disease of dogs and cats

	Dog			Cat		
	[Crt][a]		[SDMA][b]	[Crt]		[SDMA]
Stage	µmol/L	mg/dL	µg/dL	µmol/L	mg/dL	µg/dL
1	< 125	< 1.4	< 18	< 140	< 1.6	< 18
2	125–250	1.4–2.8	18–35	140–250	1.6–2.8	18–25
3	251–440	2.9–5.0	36–54	251–440	2.9–5.0	26–38
4	> 440	> 5.0	> 54	> 440	> 5.0	> 38

Source: www.iris-kidney.com/guidelines/staging.html (modified in 2023)

[a] The Crt concentrations apply to average-size dogs; those of extreme sizes may vary. The Crt concentrations for these stages are decision thresholds for the IRIS guidelines; they are not upper limits of RIs. Analytical differences and other factors may require different decision thresholds when patient results are interpreted.

[b] SDMA concentrations for these stages are based on publications and proprietary IDEXX assays and data that will not apply to all SDMA assays. IRIS gives priority to [SDMA] over [Crt] if it persistently classifies a patient in a higher stage.

Note: IRIS guidelines are for fasting samples assessed "on at least two occasions in a hydrated, stable patient." IRIS comments accompanying IRIS guidelines for each stage:

Stage 1: "Normal blood creatinine or normal or mild increase blood SDMA. Some other renal abnormality present (such as, inadequate urinary concentrating ability without identifiable nonrenal cause (in cats, not dogs), abnormal renal palpation or renal imaging findings, proteinuria of renal origin, abnormal renal biopsy results, increasing blood creatinine or SDMA concentrations in samples collected serially). Persistently elevated blood SDMA concentration (>14 µg/dL) may be used to diagnose early CKD."

Stage 2: "Normal or mildly increased creatinine, mild renal azotemia (lower end of the range lies within reference ranges for creatinine for many laboratories, but the insensitivity of creatinine concentration as a screening test means that patients with creatinine values close to the upper reference limit often have excretory failure). Mildly increased SDMA. Clinical signs usually mild or absent."

Stage 3: "Moderate renal azotemia. Many extrarenal signs may be present, but their extent and severity may vary. If signs are absent, the case could be considered as early Stage 3, while presence of many or marked systemic signs might justify classification as late Stage 3."

Stage 4: "Increasing risk of systemic clinical signs and uremic crises."

AZOTEMIA AND UREMIA

I. Definitions

A. *Azotemia* (azo = nitrogen + emia = blood) is increased concentration of nonprotein nitrogenous compounds (but not NH_4^+) in the blood that is routinely detected as increased serum or plasma [UN], [Crt], or both. Azotemia is a correct term for an increased concentration of urea or Crt. However, the pathogeneses for the increased concentrations may be different.

B. *Uremia* was classically considered to mean "urinary constituents in blood" but now typically refers to the constellation of clinical signs and nonrenal lesions reflecting renal failure (e.g., vomiting, diarrhea, pulmonary edema, coma, convulsions, ammoniacal odor of breath, oral ulcers, gastritis, and tissue mineralization).

Table 8.5. Variation in proficiency testing data for serum [Crt][a]

Sample	Species	Mean (mg/dL)	[Crt][b]			Results included	Outliers excluded
			2 SD[c] (mg/dL)	Lowest[d] (mg/dL)	Highest[d] (mg/dL)		
2021-3A	Canine	1.0	0.1	0.9	1.2	164	8
2022-4A	Canine	1.1	0.1	0.9	1.3	166	9
2016-1A	Canine	1.2	0.3	1.0	1.5	210	7
2014-3A	Canine	1.3	0.3	1.0	1.6	203	1
2013-4A	Feline	1.5	0.2	1.2	1.8	192	4
2020-2A	Feline	1.6	0.2	1.3	1.9	161	4
2010-1A	Feline	1.7	0.2	1.4	1.9	159	3
2010-4A	Canine	1.8	0.3	1.4	2.4	179	3
2012-3A	Canine	2.1	0.3	1.5	2.3	193	4
2010-3A	Canine	4.5	0.4	3.7	5.1	160	4

[a] Results were selected from canine and feline samples analyzed quarterly for the Veterinary Laboratory Association Quality Assurance Program, 2010 through 2022; most had Crt concentrations within Stage 1 IRIS guidelines. Examples spanning the range of concentrations are provided.

[b] Outlier data are not included. The number of outliers for each sample is shown in the last column to indicate how many results were even more dispersed than the provided data.

[c] SD = standard deviation rounded to the nearest 0.1 mg/dL

[d] Lowest and highest values are the lowest and highest mean values reported for each combination of method and analyzer (~30–50 combinations) that did not include an outlier. Some individual values would have been lower or higher than the provided means.

II. Azotemia classifications and disorders

A. Azotemia can occur whenever there is either decreased urinary excretion of urea or Crt or increased production of urea or Crt. The former is a much more common mechanism and the increased production by itself creates only a mild azotemia if kidneys are healthy. Two healthy kidneys have a large excess capacity to clear urea and Crt from plasma. Less urea and Crt can be excreted extrarenally.

B. Mild azotemia may not be apparent in dogs under 6 mo of age if interpreting results with respect to RIs based on adults.[21]

C. Decreased urinary excretion of urea or Crt

1. *Prerenal azotemia*: The initiating cause of abnormal urea or Crt excretion involves reduced renal blood flow.

a. Disorders (Table 8.6)

b. Pathogeneses

(1) Any process that diminishes RPF directly decreases GFR and thus decreases renal clearance of urea and Crt. The volume (stretch) receptors in the juxtaglomerular apparatus of the afferent arteriole "sense" reduced blood flow and trigger the renin–angiotensin system. Angiotensin II constricts the afferent and efferent glomerular arterioles, which further reduces glomerular perfusion and thus GFR.

(2) Hypovolemia enhances resorption of Na^+ and H_2O in PCTs, thereby promoting passive proximal tubular resorption of urea (not Crt) because the lower flow rate provides more time for resorption.

Table 8.6. Disorders and conditions that cause azotemia
Decreased urinary excretion of urea or Crt[a]
Prerenal disorders or conditions
Hypovolemia:* dehydration (including hypoadrenocorticism), shock, blood loss
Decreased cardiac output:* cardiac insufficiency, shock, hypoadrenocorticism
Shock* (hypovolemic, cardiogenic, anaphylactic, septic, neurogenic)
Renal disorders or conditions
Infectious or noninfectious inflammatory:* glomerulonephritis, pyelonephritis, tubular-interstitial nephritis (many causes)
Amyloidosis*
Toxic nephrosis* caused by hypercalcemia (many causes), oxalates (ethylene glycol, certain plants), myoglobin, grapes or raisins, lilies (cats), oak (*Quercus*) leaves or acorns, oleander (*Nerium*), heavy metals, melamine/cyanuric acid, and many drugs including aminoglycosides (e.g., gentamicin), nonsteroidal anti-inflammatory drugs (e.g., phenylbutazone), and amphotericin B
Renal ischemia or hypoxia:* poor renal perfusion, infarction (many causes)
Congenital hypoplasia, aplasia, or dysplasia
Hydronephrosis
Neoplasms (primary renal or metastatic)
Postrenal disorders or conditions
Urinary tract obstruction:* urolithiasis, urethral plugs in cats, neoplasia, prostatic disease, congenital anomalies in alpacas[22,23]
Leakage of urine from urinary tract:* trauma, neoplasms
Increased urea or Crt production (mild)
Marked upper gastrointestinal hemorrhage in dogs (↑ urea)
Postprandial of canine diets (↑ urea; ↑ Crt with boiled meat)
After strenuous exercise in dogs (↑ Crt)
Decreased clearance of Crt by a dam's placenta: ↑ [Crt] in neonatal foal (see text)
Physiologic variations in [Crt]: larger dogs > small dogs; greyhound dogs > nongreyhound dogs

* A relatively common disorder, condition, or mechanism

[a] Decreased urinary excretion involves a decreased glomerular filtration of urea or Crt except when there is leakage of urine from the urinary tract into the body.

(3) Hypovolemia also triggers release of ADH, which enhances resorption of urea (not Crt) by medullary collecting ducts. ADH enhances activity of urea-transporter A1 proteins.

(4) Azotemia is common in clinical hypoadrenocorticism, in which aldosterone deficiency impairs renal excretion of K^+; the resulting hyperkalemia can cause bradycardia and thus decreased cardiac output. Concurrently, increased renal loss of Na^+ reduces renal conservation of H_2O and sometimes results in hypovolemia. In some animals, there may be increased intestinal loss of H_2O with diarrhea or hemorrhage along with decreased H_2O intake to accentuate hypovolemia. Thus, a combination of factors can decrease renal blood flow and produce prerenal azotemia.

(5) The prerenal azotemia developing in association with severe intestinal hemorrhage is partially due to hypovolemia, which leads to decreased renal excretion of Crt and urea (see Azotemia and Uremia, Section II.D.3).

c. Decreased RPF that is severe and persistent may lead to renal hypoxia, acute renal damage, and thus acute renal failure. In such animals, the azotemia may be renal and prerenal.

2. *Renal azotemia*: The initiating cause is any renal disease that causes a major decrease in GFR. Any of the following may contribute: (i) loss of nephrons, (ii) decreased vascular patency within the kidney, (iii) decreased glomerular permeability, (iv) increased renal interstitial pressure, or (v) increased intratubular pressure.
   a. Disorders and conditions (Table 8.6)
   b. Pathogeneses
      (1) Renal disease (acute or chronic) causing the loss of at least 65–75 % of nephron functional capacity reduces the GFR sufficiently to produce azotemia. Reduced GFR causes inadequate renal excretion of urea and Crt from plasma (without sufficient compensation by intestinal processes), and thus serum [UN] and [Crt] increase.
      (2) Prerenal azotemia may be present concurrently if the animal is dehydrated or hypovolemic (a common occurrence).
3. *Postrenal azotemia*: The initiating cause of defective urea or Crt excretion from the body (not necessarily from the kidney) is distal to the nephron.
   a. Diseases or disorders (Table 8.6)
   b. Pathogenesis of obstructive azotemia
      (1) Urinary tract obstruction causes the release of vasoactive substances (prostaglandins and angiotensin) that constrict the glomerular arterioles, thus reducing RPF and diminishing the GFR; reduced GFR impairs clearance of urea and Crt.[24]
      (2) Impaired outflow causes a transient increase in intracapsular hydrostatic pressure that decreases the GFR. The pressure diminishes with time as tubular fluid diffuses into tubular cells and less filtrate is formed.
   c. Pathogenesis of azotemia caused by leakage of urine within the body
      (1) If there is leakage into the peritoneal cavity, urea and Crt enter the plasma after passive absorption through the peritoneal epithelium. The peritoneal [UN] equilibrates faster with the plasma [UN] than the peritoneal [Crt] does with the plasma [Crt] (see Chapter 19).
      (2) If there is leakage into tissue surrounding the urinary tract, urea and Crt diffuse from the extravascular to intravascular fluid and cause azotemia.
      (3) In either case, if intestinal excretion of urea and Crt does not compensate for the diminished urinary excretion, then azotemia occurs. Glomerular filtration is not reduced initially, but a calculated GFR would appear reduced if not all the urine produced during the test period were collected.
   d. Prerenal azotemia may be present concurrently if the animal is dehydrated or hypovolemic. Renal azotemia may also be present if there was sufficient reduction in renal blood flow to cause ischemic renal damage, or if there was preexisting renal azotemia.

D. Azotemia due to increased urea production, a form of prerenal azotemia
   1. Disorders or conditions (Table 8.6)
   2. Pathogenesis: Increased proteolysis generates more NH_4^+, which in turn increases the synthesis of urea by hepatocytes. Most NH_4^+ production occurs in the intestines

(see Chapter 13). If the rate of urea synthesis exceeds the rate of urea excretion, then the serum [UN] increases.

3. Experimental evidence indicates that intestinal hemorrhage can cause azotemia when there is a large amount of hemorrhage; concurrent hypovolemia also contributes to the azotemia. The azotemia due to intestinal hemorrhage results from two processes: (i) increased urea production because the marked degradation of hemoglobin and other blood proteins increases NH_4^+ delivery to hepatocytes, and (ii) decreased GFR associated with the hypovolemia.
   a. Feeding whole blood at a rate nearly equal to 10 % of a dog's blood volume increased the [UN] by about 10 mg/dL, whereas feeding whole blood at a rate nearly equal to 25 % of a dog's blood volume increased the [UN] by about 20–30 mg/dL. The peak azotemia occurred by 12 h, and nearly all UN concentrations returned to baseline values by 24 h.[25]
   b. Withholding H_2O for 24 h and feeding whole blood to six dogs at a rate near one-third of a dog's blood volume increased UN concentrations by increments of 7–37 mg/dL in individual dogs. Peak concentrations occurred 4–7 h after feeding and returned to baseline values by 24–36 h. Removing 52–76 % of the blood volumes of seven dogs while restricting access to H_2O decreased blood pressures and caused concurrent azotemias. Concentrations of UN were increased 2–25 mg/dL in individual dogs from 12–24 h after bleeding. The combination of bleeding (near 50 % blood volume) during several hours and feeding blood (near 30 % blood volume) caused greater increases in [UN] (20–67 mg/dL increases in ten dogs). The authors concluded that hemorrhage causing decreased blood pressure, feeding a large amount of blood, or a combination of these processes can create azotemia.[26]
   c. In a retrospective study in which 52 dogs with upper gastrointestinal hemorrhage (hematemesis and/or melena) were compared to 52 dogs without upper gastrointestinal hemorrhage, the median [UN] was 14 mg/dL greater in the hemorrhage group. Also, the serum UN:Crt ratio was greater in the hemorrhage group (median 28 versus 14). However, this study did not establish whether the azotemias associated with gastrointestinal hemorrhage were caused by increased urea production, decreased urea excretion, or both, as the authors did not determine whether the hydration status of the hemorrhage group was similar to that of the control group, and the presence or absence of anemia was not reported. In the data provided, some dogs with hemorrhage were not azotemic, and other dogs had increased [Crt] (up to 3.1 mg/dL).[27]
   d. In experimental conditions involving people, it was shown that the degree of azotemia after consumption of blood is highly dependent on either the presence of hypovolemia or impaired renal function.[28]
4. Postprandial increases in [UN] have been reported in healthy dogs. Mild azotemia and mean peak postprandial increases of 1–23 mg/dL have been reported 4–8 h after ingestion of various diets, the greatest increases occurring with meat ingestion.[29–31] This cause of azotemia is excluded when blood is collected from a fasted or anorexic dog.
5. Increased protein catabolism associated with systemic disorders (e.g., infections, neoplasms, malnutrition) is frequently stated to provide sufficient NH_4^+ to increase urea production and produce azotemia. Documentation of this mechanism was not found.

E. Azotemia due to increased Crt production, a form of prerenal azotemia (mild)
   1. Postprandial increases in [Crt] have been reported in healthy dogs with some diets but not others. This cause of azotemia is excluded when blood is collected from a fasted or anorexic dog.
      a. Minor increases in [Crt] were reported in dogs after they ate cooked meat.[29] The mean [Crt] for six dogs 1–4 h after ingesting boiled meat was 1.4 mg/dL compared to 0.9 mg/dL before ingestion. Mean [Crt] actually decreased 0.1–0.2 mg/dL after raw meat or soft-moist feedings. The increase in [Crt] after eating boiled meat might indicate increased ingestion of Crt; cooking converts about 20–65 % of muscle creatine to Crt.[32] The minor decreases after eating raw meat or the soft-moist food were not explained. The report did not mention the presence or absence of lipemia in the postprandial samples, but the [Crt] was measured by an alkaline picrate kinetic assay which is relatively unaffected by lipemia.
      b. In other studies, dogs fed various meals (dry, semimoist, canned, pelleted) had little increase in [Crt] 4 h after ingestion, the greatest mean increase being 0.1 or 0.2 mg/dL, depending on the assay.[30,31]
   2. Postexercise increases in [Crt] have been reported in sled dogs soon after 30–35 min of strenuous exercise; in one study, the mean increase was 0.4 mg/dL,[33] and most increases were < 50 % in another study.[34] However, post-race decreases in [Crt] have been reported in endurance sled dogs,[35] and increased [Crt] was not found after a 36 h rest period during a Yukon race[36] or in healthy beagles after a 60 min run.[37]

III. Guidelines for azotemia differentiation (Table 8.7)
   A. The cause of azotemia may be multifactorial. Prerenal disorders (e.g., hypovolemia) and postrenal disorders (e.g., obstruction) can cause acute renal disease and thus acute renal failure. Animals with renal failure may also be hypovolemic. Accordingly, at the time of presentation, an animal's azotemia may be the product of renal and extrarenal factors.
   B. The major laboratory criterion for differentiating azotemias involves the USG_{ref}. However, the diagnostician must consider the renal and extrarenal factors that may influence an animal's ability to concentrate and dilute urine.
      1. If azotemia is exclusively prerenal and related to decreased GFR, the USG_{ref} is expected to be > ~1.030 in dogs (see Table 8.7 for other species) because the kidneys are being stimulated to conserve H_2O.
      2. If the USG_{ref} is below these values in azotemic animals and there is no evidence of increased urea production (e.g., gastrointestinal hemorrhage), there is impaired renal concentrating ability, but it may be due either to primary renal disease (renal azotemia) or to extrarenal disease (prerenal azotemia with impaired concentrating ability unrelated to primary renal disease).
         a. Renal disease
            (1) The USG_{ref} is often 1.007–1.013 because of impaired tubulointerstitial function associated with nephron damage.
            (2) The USG_{ref} may be > 1.013 but still inappropriately low (less than the appropriate species USG_{ref} cutoffs for adequate concentration in prerenal azotemic states) if:
               (a) The renal disease impairs glomerular function more than tubulointerstitial function (especially in cats or in any animal with acute renal failure).

Table 8.7. Major criteria used to differentiate azotemia caused by decreased GFR

Type	Expected USG_{ref}[a]	Urine volume	History, physical exam, or other information
Prerenal	> 1.030 (dog) > 1.040 (cat) > 1.025 (horse, cattle) > 1.018? (llama, alpaca)	↓[a]	Decreased GFR due to dehydration, acute hemorrhage, shock, or decreased cardiac output
Renal	1.007–1.013[b]	Usually ↑ May be ↓	Other UA findings, electrolyte changes, or anemia that are suggestive of renal disease (acute or chronic)
Postrenal	?[c]	↓[c]	Dysuria, enlarged or ruptured urinary bladder, urine in abdomen

[a] These criteria assume there are not extrarenal disorders that are affecting the renal concentrating ability and urine volume.

[b] Assuming there are not substances (such as protein and glucose) that interfere with USG_{ref} assessment of the urine solute concentration; a USG_{ref} of 1.014–1.030 also reflects impaired concentrating ability in a dehydrated or azotemic dog.

[c] USG_{ref} depends on the animal's hydration status and presence or absence of concurrent renal disease. During postobstructive diuresis, the USG_{ref} is low and urine volume is increased.

Note: Prerenal, renal, and postrenal disorders may occur independently or in combinations.

(b) High urine concentrations of protein or glucose affect the USG_{ref} such that the USG_{ref} overestimates urine osmolality (see Physical Examination of Urine, Section III.B.5).

(c) Plasma osmolality is increased, so urine osmolality is greater than expected despite failure to dilute or concentrate the filtrate.

d. Extrarenal causes

(1) Azotemia develops from hypovolemia.

(2) Inappropriate renal concentrating ability is caused by one or more of the following:

(a) Epithelial cells of the collecting ducts are not responsive to ADH (nephrogenic diabetes insipidus). This may occur, for example, because of hypercalcemia.

(b) Solute overload (too much solute entering the LoH as occurs with osmotic diuresis) causes a high flow rate and decreases resorption of tubular fluid.

(c) Decreased medullary hypertonicity because of the following:

(i) Prolonged hyponatremia or hypochloremia

(ii) Blocked Na^+ and Cl^- transport (e.g., loop diuretics)

(iii) Decreased urea production because of hepatic disease

(iv) Solute overload or prolonged diuresis

UREA NITROGEN (UN) CONCENTRATION IN SERUM OR PLASMA

I. Physiologic processes or concepts regarding urea (Figure 8.6)

A. Urea is formed within hepatocytes. The major route of excretion is renal.

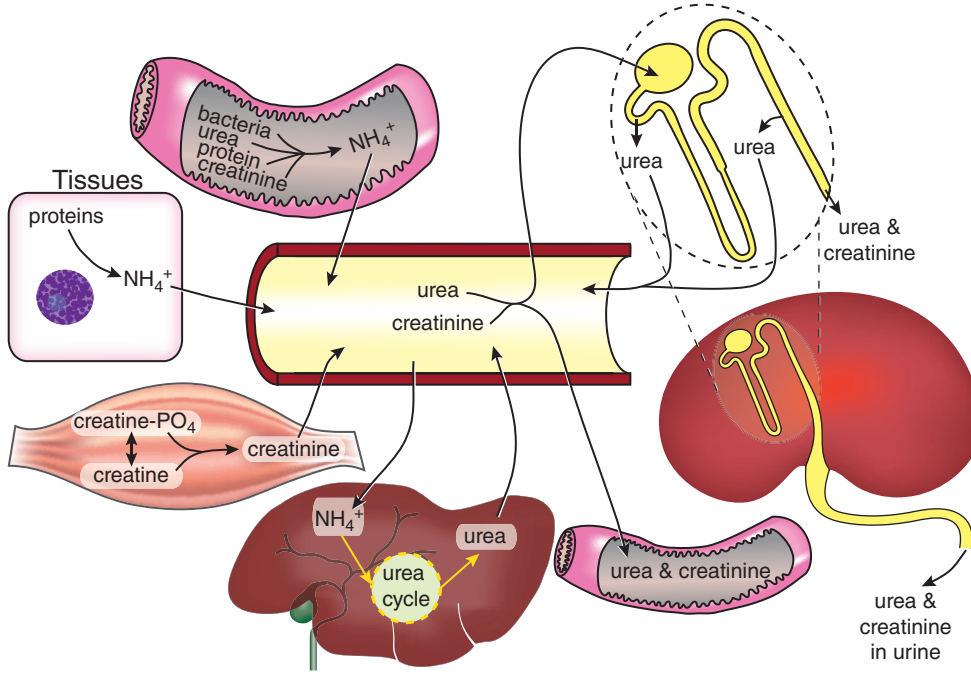


Figure 8.6. Physiologic processes or concepts concerning urea and Crt.
Urea synthesis occurs in hepatocytes via the urea cycle, which is one method of incorporating NH_4^+ into molecules for excretion of excess NH_4^+ that is formed in tissues or intestine. After urea passively enters plasma from hepatocytes, it has two possible fates.

- Urea passes freely across the GFB and is excreted in urine or resorbed by renal tubules. Urea resorption is enhanced by H_2O resorption in PCTs. In the medullary collecting ducts, increased ADH activity enhances urea-transporter activity and thus increases urea resorption.
- Urea enters the intestinal tract of monogastric mammals (via the blood or the biliary system) where it is degraded by enteric bacteria (with urease), passively absorbed into portal blood, or excreted in feces. In cattle, urea enters the rumen (via saliva and blood), where it is degraded to NH_4^+.[38]

Crt is the product of creatine (not creatinine) degradation. Creatine phosphate serves as a ready source of PO_4 that is used to generate ATP for muscle contractions (creatine + ATP ↔ creatine PO_4 + ADP). Crt enters the plasma after the degradation of creatine or creatine PO_4 in myocytes of the animal or of dietary meat. Crt is excreted from the body via the kidneys and intestine.

- Crt passes freely across the GFB; it is not resorbed by tubules. Small quantities may be secreted by functional PCTs when there is increased plasma [Crt].
- Crt is also degraded and excreted in feces of people[39,40] and in saliva of cattle.[38] Alimentary tract excretion is suspected to occur in dogs, cats, and horses, as Crt is diffusible across most cell membranes.

B. Urea freely diffuses across cell membranes, but relatively slowly because it is a polar molecule. Diffusion of H_2O into and out of blood or into and out of cells is accompanied by urea. Where rapid movement of urea is necessary, urea transporters facilitate its movement; this occurs in medullary collecting ducts, the vasa recta, RBCs, and hepatocytes.[9]

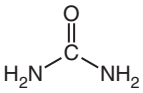
C. Extrarenal excretion of urea varies among species, probably because of physiologic and pathologic processes.

1. Urea that enters the intestine can undergo spontaneous degradation or be split by urease-containing bacteria to form NH_4^+. In people, about 20–25 % of radiolabeled urea was eliminated nonrenally in a manner that was attenuated by the oral administration of neomycin.[41]
2. In dogs with decreased renal function, the extrarenal excretion of urea did not increase to a statistically significant degree, except as a percentage of total plasma urea clearance, which was reduced.[42]
3. In cattle and other ruminants, urea enters the rumen (via saliva and blood), where it is degraded to NH_4^+.[38]
4. In llamas, the amount of urea excretion via the digestive tract appears to be greater than that in domestic ruminants.[43] Most of the urea is degraded in forestomach compartment 1.

II. Analytical concepts

A. Terms and units

1. Current clinical assays measure [urea] in serum, plasma, or whole blood, and some laboratories report [urea] directly. In many, though, the clinical custom is to express [urea] in terms of the nitrogen content in the urea, i.e., as urea nitrogen (UN).
   a. Urea has a M_r of 60, so it weighs 60 g/mol. It is composed of one carbon, one oxygen, two nitrogen, and four hydrogen atoms. Therefore, 1 mol of urea contains 28 g of nitrogen. Accordingly, a [urea] of 1 mmol/dL = a [urea] of 60 mg/dL = a [UN] of 28 mg/dL.



Urea

   b. [Urea], reported as the concentration of UN, is commonly referred to as *BUN* (blood urea nitrogen), although usually serum, not whole blood, is assayed for *SUN*. Because urea diffuses across cell membranes, the extracellular [UN] and intracellular [UN] in blood are usually similar. Therefore, the [UN] in serum ≅ [UN] in blood ≅ [UN] in plasma (i.e., SUN ≅ BUN ≅ PUN).
2. Unit conversion
   a. mg/dL of UN × 0.3570 = mmol/L of urea (SI unit)[44]
   b. mg/dL of urea × 0.1665 = mmol/L urea

B. Sample

1. Serum or plasma may be used in most spectrophotometric assays.
2. Urea is stable in serum or plasma for at least 1 d at room temperature, several days at 4–6 °C, and at least 2–3 mo when frozen.

C. Principles of urea assays

1. Current urea assays typically use a reaction catalyzed by urease in which urea is hydrolyzed to form either NH_3 or NH_4^+. The amount of NH_3 or NH_4^+ formed is measured by an indicator system and reflects the sample's urea content.
2. The concentration of either NH_3 or NH_4^+ in the original sample is typically negligible unless contaminated with NH_4^+ (e.g., with quaternary ammonium compounds).
3. Fluoride inhibits urease activity, so NaF tubes should not be used to collect blood samples for a urease assay.
4. Free hemoglobin (hemolysis), lipemia, or hyperbilirubinemia may interfere with some assays: the manufacturer's insert for the assay should be consulted.

Table 8.8. Disorders and conditions that cause decreased serum or plasma [UN]
Disorders that cause decreased urea synthesis
Hepatic insufficiency:* hepatocellular disease, portosystemic shunts, hepatic microvascular dysplasia
Urea cycle enzyme deficiencies
Disorders that cause increased renal excretion of urea
Disorders that cause impaired PCT resorption of urea:* glucosuria
Central or nephrogenic diabetes insipidus
Disorders that increase GFR (e.g., hyperthyroidism in cats)

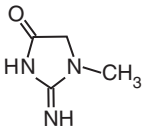
* A relatively common disorder, condition, or mechanism

D. Increased [UN] in serum or plasma (azotemia) (Table 8.6)
E. Decreased [UN] in serum or plasma (Table 8.8)
   1. Disorders that decrease urea synthesis
      a. Hepatic insufficiency (see Chapter 13)
         (1) Extensive hepatocellular disease that markedly reduces functional hepatic mass (> 80 % loss) and thus sufficiently decreases urea synthesis causing decreased plasma [UN] and potentially increased plasma [NH_4^+]
         (2) Portosystemic shunt (congenital or acquired)
            (a) Less NH_4^+ is delivered to the hepatocytes from the intestines via the portal vein.
            (b) There is less uptake of NH_4^+ by hepatocytes because of decreased functional hepatic mass due to atrophy, necrosis, or fibrosis.
      b. Urea cycle enzyme deficiencies (congenital, extremely rare)
   2. Disorders that increase renal excretion of urea
      a. When less H_2O is resorbed in the PCTs (e.g., because of glucosuria or expanded extracellular volume), less of the filtered urea is resorbed in the PCTs because the resorption of H_2O creates the concentration gradient for urea resorption.
      b. In central and nephrogenic diabetes insipidus, reduced ADH activity or response to ADH in the medullary collecting ducts decreases resorption of urea and H_2O.
      c. Increased GFR promotes renal clearance of urea and may reduce plasma [UN]. This can mask a decrease in functional glomeruli as can occur in cats with hyperthyroidism and concurrent renal disease; treatment of hyperthyroidism can unmask glomerular disease, i.e., cause azotemia. Treatment effects on [SDMA] are inconsistent and can be discordant with [Crt], perhaps because of thyroxine effects on [SDMA].[45]
   3. Consequence: The amount of urea in the renal interstitial fluid may diminish. Because about 50 % of the medullary hypertonicity is normally due to urea, the urea deficit may contribute to a reduced concentration gradient, impaired renal concentrating ability, and thus polyuria.

CREATININE (Crt) CONCENTRATION IN SERUM OR PLASMA

I. Physiologic processes or concepts regarding Crt (Figure 8.6)
   A. Crt is formed within muscle from the degradation of creatine and creatine phosphate (not "creatinine phosphate").
   B. Most studies suggest that inter-individual biological variation of serum [Crt] is great enough compared to biological variation within individuals to make within-subject comparisons (change from an established baseline, not single point) more useful than population-based RIs alone.
   C. Crt diffuses across most cell membranes, and thus H_2O moving in and out of blood or in and out of cells is accompanied by Crt. The major exception for this free movement is in the renal tubules.
   D. The amount of extrarenal excretion of Crt is not firmly established among species, and probably varies because of physiologic and pathologic processes.
      1. In people, Crt is found within gastric and intestinal fluid and most likely is degraded within the intestinal fluid. In people with reduced renal function, 16–66 % of the endogenously formed Crt is estimated to be excreted extrarenally.[40] The enhanced extrarenal excretion may be due to increased intestinal degradation of Crt by bacterial creatininase.[46]
      2. In dogs with reduced renal function, there was no significant increase in extrarenal excretion of Crt, but the percentage of Crt excretion that was extrarenal increased mildly when GFR was markedly decreased.[42]

II. Analytical concepts
   A. Terms and units
      1. Crt has a M_r of 113, about twice that of urea.
      2. Unit conversion: mg/dL × 88.4 = µmol/L (SI unit)[44]



Creatinine

   B. Sample
      1. Serum or plasma may be used in most spectrophotometric assays.
      2. Crt in serum (not whole blood) is stable for up to 4 d at room temperature and longer (for 1–3 mo) when stored at −20 °C.[32]
   C. Principles of Crt assays
      1. In Jaffe or modified Jaffe assays, Crt reacts with picric acid to form a colored complex. In Jaffe assays, a positive interference can be created by nonCrt chromogens including proteins, glucose, acetoacetate, β-hydroxybutyrate, ascorbic acid, pyruvate, and cephalosporins.[47]
         a. There is less interference in modified Jaffe assays and also less interference in kinetic assays compared to endpoint assays. In a study using domestic animal sera in a kinetic Jaffe assay, acetone, cephalosporins, and glucose caused positive biases, whereas acetoacetic acid, bilirubin, and lipid produced negative biases.[48]
         b. A [bilirubin] of 10 mg/dL may reduce the [Crt] about 1 mg/dL.[49] Deproteinization of sera prior to the Jaffe reaction reduces the interference. Some Jaffe assays remove bilirubin interference by including potassium ferrocyanide as a reagent that converts bilirubin to biliverdin prior to the picric acid reaction.[50]
         c. In some assays (e.g., Roche Diagnostics reagents for the Hitachi or Cobas instruments), 0.3 mg/dL is subtracted from the assay result to remove the positive interference of serum proteins. However, this correction factor assumes normoproteinemia. With hyperproteinemia, the correction factor is inadequate;

with hypoproteinemia, the correction factor is excessive. Some instruments (Beckman Coulter) provide an option to either apply or not apply a correction factor.

2. In enzymatic spectrophotometric assays, Crt reacts with creatininase to form creatine which is acted on by creatinase to form sarcosine and urea. Sarcosine oxidase acts on sarcosine to produce H_2O_2, which reacts with an indicator to generate a colored dye. Enzymatic methods are not affected by nonCrt chromogens and may yield lower values than methods using Jaffe's reaction.[32]
3. Some blood-gas analyzers and point-of-care instruments use the creatininase reaction to produce H_2O_2, which then is detected electrochemically.
4. Variations in Crt assays result in disagreement among assays (Table 8.5).

III. Variations in [Crt] in healthy dogs: Differences in body weight and differences in muscle mass are associated with minor differences in [Crt].

A. Differences in [Crt] were found among 317 clinically healthy, purebred dogs; [Crt] was measured with an enzymatic Crt assay.[51] The reported [Crt] values represent the mean ± 2 SD: mini dogs (< 10 kg), 0.5–1.0 mg/dL; medium dogs (11–25 kg), 0.6–1.2 mg/dL; maxi dogs (26–45 kg), 0.6–2.0 mg/dL; giant dogs (> 45 kg), 0.9–1.8 mg/dL (significant figure rules have been applied to the reported [Crt]). It was not stated if any of the dogs were obese or lean.
   1. Clinical laboratories likely will not provide canine RIs based on body weights; a dog's body weight (especially for the smallest dogs) should be considered when a patient's [Crt] is near an upper reference limit.
   2. Lower [Crt] in smaller dogs may be related to decreased muscle mass. However, another study documented that the smallest dogs had greater estimated GFR values than larger dogs.[52] Extrarenal Crt excretion in dogs of various sizes has not been reported.

B. When data from 10 healthy greyhound dogs and 10 size-matched nongreyhound dogs were compared, the [Crt] and GFR values in the greyhounds were greater: 1.8 ± 0.1 mg/dL and 3.0 ± 0.1 mL/min/kg, respectively, versus 1.5 ± 0.1 mg/dL and 2.5 ± 0.2 mL/min/kg in the nongreyhound dogs. The authors suggested the greyhound dogs have greater Crt production because of their muscle mass. Differences in extrarenal excretion of Crt were not explored.[53] In another study, [Crt] in 30 greyhound dogs was 1.2 to 1.9 mg/dL whereas [Crt] in 30 nongreyhound dogs was 0.8 to 1.7 mg/dL.[54]

C. The [Crt] measured in 33 1- to 8-yr-old sighthounds (subgroup of 111 Afghan hound, saluki, whippet, greyhound, borzoi, and Scottish deerhound dogs) ranged from 81 to 195 µmol/L (0.9–2.2 mg/dL) with a mean of 111 µmol/L (1.3 mg/dL), whereas the RI for most dogs was < 100 µmol/L (1.1 mg/dL).[55]

D. In a study with 38 beagles, there was a weak positive correlation (R^2 = 0.29) between serum [Crt] and lean body mass, with [Crt] ranging from about 0.4 to 1.0 mg/dL in dogs with lean body masses of approximately 5–13 kg.[56]

E. Total plasma Crt mass (mg), determined as the product of plasma [Crt] and measured total plasma volume, was quite consistently proportional to the mass (weight) of skeletal muscle removed from four lean, nonazotemic, exsanguinated dogs weighing 13.6–19.1 kg.[57] However, the relationship between total body weight and plasma [Crt] rather than plasma Crt mass is more complex because it is affected by nonmuscle mass and blood volume.

IV. Increased [Crt] in serum or plasma (azotemia) (Table 8.6)

A. [Crt] is typically increased by pathologic processes that cause decreased GFR; the initiating process may be prerenal, renal, or postrenal, with multiple processes commonly contributing.

B. Increased Crt production and release from damaged myocytes could contribute to increased serum [Crt] when renal function is impaired (e.g., myoglobinuric nephrosis secondary to rhabdomyolysis in horses), but Crt is quickly cleared from plasma if renal function is adequate.

C. Neonatal foals can have an increased serum [Crt] if born to dams with dysfunctional placentas that prevent normal clearance of fetal Crt by placental blood. In contrast to congenital renal failure, this azotemia should diminish quickly after birth (by 50 % if nursing well and hydrated) and resolve over several days because Crt will be excreted in urine.[58] In a study of 28 neonatal foals with similar rapid resolution of increased [Crt] unaccompanied by laboratory evidence of acute renal failure, placental lesions were reported in only 5 foals, whereas 20 foals had neonatal encephalopathy that may have been caused by prolonged hypoxia related to dystocia or premature placental separation.[59] The cause of increased [Crt] was not clear. It was considered "spurious hypercreatininemia," but in the context of laboratory data, *spurious* and *factitious* refer to false data, i.e., pseudohypercreatininemia without an actual increase in [Crt]. Evidence to indicate false increases was lacking. One report suggests that similarly affected foals may have increased serum [SDMA] as well as [Crt], thus supporting insufficient renal clearance.[60]

D. The [Crt] of clinically healthy Birman cats may exceed the upper reference limit of routine feline RIs for [Crt].[61]

V. Decreased [Crt] in serum or plasma

A. A decreased [Crt] in serum or plasma is probably not clinically significant. Animals with a decreased muscle mass tend to have a lower [Crt], and the presence of hypoproteinemia could yield a slightly lower [Crt] (see Creatinine Concentration in Serum or Plasma, II.C.1.c). In addition, conditions such as feline hyperthyroidism that increase GFR may reduce [Crt] and mask a concurrent decrease in functional glomeruli that may become evident after effective hyperthyroidism treatment.

B. In 21 dogs with portosystemic vascular anomalies, most had a [Crt] of 0.2–0.4 mg/dL (lower reference limit 0.5 mg/dL), most had an increased measured GFR, and most were hypoproteinemic based on sums of the reported concentrations of albumin and globulins; total protein concentrations were not reported.[62] Months after surgical ligation of shunts, fewer dogs had low [Crt] and fewer dogs were hypoproteinemic.

1. Findings support that the increased GFR caused an increased renal clearance of Crt and thus contributed to lower Crt concentrations.
2. Other factors may have contributed. The [Crt] was measured on a Hitachi 911 and thus the protein-correction factor of 0.3 mg/dL (see Principles of Crt Assays above) could have contributed to a falsely lower [Crt]. Additionally, the authors could not exclude contributions related to age (lower in puppies at the time of ligation), hepatic insufficiency (reduced creatine synthesis), or decreased muscle mass (impaired growth).

C. If the lower reference limit for serum [Crt] were near the detection limit of a Crt assay, documenting a true decrease would be difficult.

UREA NITROGEN (UN) CONCENTRATION VERSUS CREATININE (Crt) CONCENTRATION IN SERUM OR PLASMA

I. Concepts
   A. In most mammals, increases in [Crt] and [UN] generally parallel each other, and thus the same information can usually be gained from either value alone. In horses, [Crt] tends to be more sensitive than [UN] to decreases in GFR, probably because of urea excretion into the alimentary tract. Similarly, in ruminants and llamas[43] with renal failure, urea excretion into the alimentary tract may cause disproportionate increases in [Crt].
   B. In theory, [Crt] is a better indicator of decreased GFR compared to [UN] because the quantity of Crt presented to the kidneys is more constant and Crt is not resorbed by the tubules, whereas urea production is not constant, and urea is resorbed by renal tubules.
      1. Hypovolemia increases urea resorption in the tubules because of decreased flow rate in the tubules, which allows more time for urea diffusion, and ADH promotes urea resorption in the collecting ducts.
      2. Increased protein in intestinal contents (high protein diet or massive intestinal hemorrhage) leads to increased generation of NH_4^+ and subsequently increased urea synthesis if digestive and absorptive processes are functioning.
      3. Intestinal excretion of urea and Crt may influence serum [UN] and [Crt].

II. Serum UN:Crt ratio
   A. Clinical observations have suggested that a serum UN:Crt ratio can help differentiate prerenal and renal azotemias as follows:
      1. An increased serum [UN] and concurrent serum [Crt] within RI is most likely a prerenal azotemia.
      2. If serum [Crt] is increased proportionately to or more than serum [UN], then the azotemia is probably renal or postrenal.
   B. Published serum UN:Crt ratios for azotemic dogs[63] are listed in Table 8.9. The authors concluded the following:
      1. The most severe azotemias occur with renal or postrenal azotemias, whereas the greatest serum UN:Crt ratios are found in prerenal azotemias. However, renal azotemia cannot be consistently differentiated from extrarenal azotemias by means of the serum UN:Crt ratio because of the overlapping values.

Table 8.9. UN:Crt ratios in azotemic dogs and cats

	Prerenal ($n = 6$)		Renal ($n = 78$)		Postrenal ($n = 17$)	
	Average	Interval	Average	Interval	Average	Interval
[UN] (mg/dL)	89	21–183	140	38–470	194	85–340
[Crt] (mg/dL)	2.4	0.4–6.4	4.9	1.2–11.1	9.1	1.3–20
UN:Crt ratio	55.3	10–260	29.8	7–102	30.5	12–128

Source (adapted from): Finco and Duncan[63]

2. Concentrations of UN and Crt in serum should be regarded as crude indices of renal function because both lack diagnostic sensitivity and specificity for renal dysfunction caused by renal disease.

RENAL CREATININE (Crt) CLEARANCE RATE

I. *Renal Crt clearance rate*, which is the rate at which Crt is cleared from plasma by the kidneys, is a good estimate of GFR in domestic animals but is not equivalent to GFR. A decreased Crt renal clearance rate (if a valid result) indicates the animal has a decreased GFR. However, the cause of the decreased GFR can be prerenal, renal, or postrenal.

II. Renal Crt clearance rate formula (Eq. 8.1)

$$\text{Creatinine clearance rate} = \frac{[\text{Crt}]_u}{[\text{Crt}]_s} \times \text{volume}_u \div \text{time} \div \text{bw} \qquad (\mathbf{8.1})$$

$[\text{Crt}]_u$ = [Crt] in collected urine during a timed collection period
$[\text{Crt}]_s$ = [Crt] in serum from blood collected during the timed urine collection period
volume_u = urine volume (in mL) collected during a timed collection period
time = length of time (in min) during the urine collection period
bw = body weight (in kg) of animal
units = mL/min/kg = mL of plasma that were cleared of Crt/min/kg

III. Endogenous renal Crt clearance rate
   A. Indications
      1. To assess GFR in nonazotemic, nondehydrated animals that are suspected of having renal disease, often because they are polyuric (and without azotemia or with only mild azotemia that might not reflect disease)
      2. To obtain a more objective assessment of the degree of impaired GFR in azotemic animals, which may be helpful in predicting prognosis or monitoring response to therapy
   B. Basics of the procedure:[64] Adequate hydration must be established or confirmed. The urinary bladder must be emptied completely. All urine produced during a specified period (20 min to 24 h) is collected via a metabolism cage or catheterization, and the volume is recorded. A blood sample for serum [Crt] is collected during the urine collection period. The urine is mixed well, and the urine [Crt] is measured. It is critical that all urine produced is collected (with no spillage and minimal evaporation), especially when shorter collection periods are used.
   C. Potential technical problems include failure to empty the bladder completely before the procedure, failure to collect all the urine formed during the collection period, decreased GFR due to undetected patient dehydration, and assay interferents (e.g., effects of nonCrt chromogens).
   D. RIs: dogs, 3.7 ± 0.77 mL/min/kg (mean ± SD),[65] 3.64 ± 0.10 mL/min/kg (mean ± standard error of the mean);[64] cats, 2.94 ± 0.32 mL/min/kg (mean ± SD),[18] 2.56 ± 0.61 mL/min/kg (mean ± SD);[66] horses, 1.48 ± 0.043 mL/min/kg (mean ± standard error of the mean),[67] 1.92 ± 0.51 mL/min/kg (mean ± SD)[68]

E. Endogenous renal Crt clearance rate is generally more sensitive than serum [Crt] for detecting decreased GFR because it is a direct assessment of renal excretion of Crt. Serum [Crt] depends on Crt production, renal excretion of Crt, and intestinal Crt excretion.

IV. Exogenous renal Crt clearance rate[69]
- A. Basics of the procedure: It is the same as the endogenous procedure except that Crt is injected into the patient and the urine collection period is routinely short.
- B. Advantages: There is increased plasma [Crt] and therefore increased challenge to the kidneys, so it is a better assessment of GFR. The procedure minimizes effects of assay interferents.
- C. Disadvantages: It may increase the percentage of excreted Crt from tubular secretion. There is a lack of standardization of methods.

RENAL IOHEXOL CLEARANCE RATE

I. Iohexol is an iodinated compound that can be used for contrast radiography when administered intravenously. It also can be used to assess GFR as it is not bound to plasma proteins, is cleared from plasma via glomerular filtration, is neither absorbed nor secreted by renal tubules, and is not degraded by the body.[70] The procedure is done on a well-hydrated but fasted animal following specific laboratory instructions. Important information used to determine clearance includes the patient's weight, the volume and concentration of the administered iohexol, and the precise times of iohexol administration and blood collections.

II. Iohexol clearance assesses the rate of renal iohexol clearance from plasma after an intravenous injection. In contrast, renal Crt clearance assesses the renal clearance of Crt from plasma by establishing the plasma concentration of Crt presented to the kidneys and the amount of Crt excreted in urine.
- A. The main advantage of assessing GFR via iohexol plasma clearance is that urine collection is not needed, whereas it is needed to determine renal Crt clearance.
- B. The main disadvantage of assessing GFR via renal iohexol clearance is that special analytical methods are needed to measure the plasma or serum iohexol concentrations. Methods of measuring iohexol concentrations include ultraperformance or high performance liquid chromatography, micellar electrokinetic capillary chromatography, high-performance capillary electrophoresis, atomic-emission spectroscopy, X-ray fluorescence, and spectrophometric analysis of iodide content.[71–74] There are reports that the various methods lack analytical agreement, which leads to differences in the calculated clearance values.[72,75]
- C. When compared to exogenous renal Crt clearance rates, iohexol clearance rates mildly overestimated GFR when a 1-compartment model was used to compute iohexol's clearance rate.[76] Using a 3-compartment model for iohexol clearance in 10 healthy horses, iohexol clearance was significantly greater (mean = 2.38 mL/min/kg) than exogenous Crt clearance (mean = 2.11 mL/min/kg).[77]

III. After establishing a good state of hydration, the procedure consists of an intravenous injection of iohexol (time 0) which is followed by subsequent timed blood collections. After the iohexol concentrations are measured in the collected plasma or serum samples, the values are used to calculate the iohexol clearance rate from plasma, and thus the GFR. In studies of cats or dogs, samples have been collected at 5, 20, 40, 60, 80, 100, 120, 150, 180, and 240 min.[78,79] For

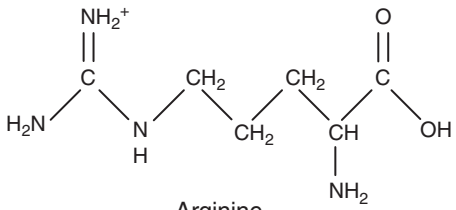
equine studies, samples have been collected at 5, 20, 40, 60, 120, 240, and 360 min.[77] Estimates of GFR have been obtained by more-practical 2-sample (3 and 5 h),[80] 4-sample (2, 3, 4, and 5 h),[80] and 3-sample protocols (2, 3, and 4 h) in cats,[81,82] a 3-sample (2, 3, and 4 h) protocol in dogs,[52] and a 3-sample (5, 30, and 90 min) protocol in horses.[83] Based on a study comparing 1-, 2-, and 3-sample protocols to a 5-sample protocol (5, 15, 60, 90, and 180 min) for use in dogs, reduced sampling protocols that included the 180-min sample performed best.[84]

IV. GFR was estimated in 118 healthy dogs of four different body-weight groups using a 3-sample iohexol method.[52] There was considerable overlap in the renal iohexol clearance rates among groups, but the rates were greater (1.54–4.25 mL/min/kg) in the 29 smallest dogs (1.8–12.4 kg) than in the three groups of larger dogs (1.29–3.50 mL/min/kg in 13.2–25.5 kg dogs, 0.95–3.36 mL/min/kg in 25.7–31.6 kg dogs, and 1.12–3.39 mL/min/kg in 32.0–70.3 kg dogs).

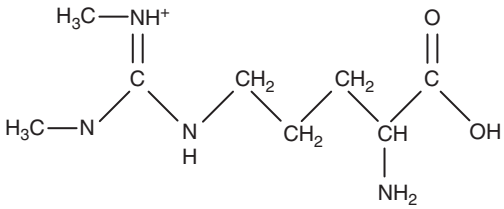
SYMMETRIC DIMETHYLARGININE (SDMA) CONCENTRATION IN SERUM OR PLASMA

I. Physiologic concepts

A. Arginine is a semi-essential amino acid for people. In cats, it is an indispensable amino acid, and dogs have an intermediate need; evidence suggests that it is essential for horses.[85] Proteolysis of arginine-containing proteins can create three methylated analogs of arginine: monomethylarginine, asymmetric dimethylarginine, and SDMA. The major enzyme forming SDMA is protein arginine methyltransferase 5, a widely expressed transcription regulator that supports cell survival.



Arginine



Symmetric dimethylarginine (SDMA)

B. The major method of eliminating SDMA from plasma is via renal excretion; there may be a small percentage removed by enzymatic degradation (Figure 8.7).[86] Because of this, increased serum [SDMA] has been used as a marker of decreased GFR in people since the early 1990s.[86]

C. Breed differences in plasma [SDMA] have been recognized but are not explained. Many breeds have not been evaluated.

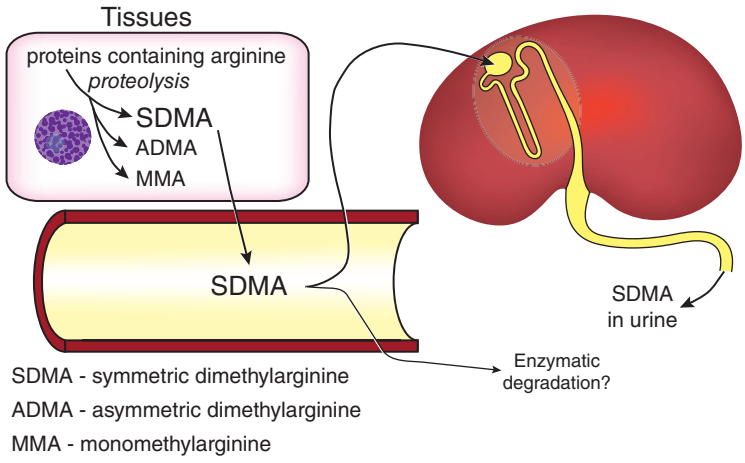


Figure 8.7. Physiologic processes or concepts concerning SDMA. SDMA is one of three products of arginine degradation that is produced during the proteolysis of arginine-containing proteins. After it diffuses into plasma, SDMA easily passes through the GFB and is excreted in urine. A small amount of SDMA may be removed from plasma via enzymatic degradation.

1. The mean serum [SDMA] for greyhounds (13.1 µg/dL) was greater than that of a group of dogs excluding sighthounds (10.2 µg/dL).[87] No significant breed differences in [SDMA] were found in 38 4- to 10-yr-old German or English (not specified which) pointers, Cairn terriers, and Cavalier King Charles spaniels.[88]
2. Birman cats had a greater median serum [SDMA] (12.0 µg/dL) than other cats (9.7 µg/dL).[61]

D. Age-related differences have been reported. Neonatal thoroughbred foals < 36 h of age had SDMA concentrations that were about five times adult values, whereas foals aged 2–6 mo had intermediate concentrations.[60] Based on proprietary data, a mildly higher RI for [SDMA] has been suggested for puppies, but values in greyhound puppies 3–8 mo of age were similar to those of adults.[89]

II. Analytical concepts

A. Measurement of [SDMA] in veterinary species can be done by high-performance liquid chromatography and gas or liquid chromatography–mass spectrometry. Liquid chromatography–mass spectrometry has been regarded as the reference standard when an appropriate internal standard is used. Development of automated immunoassays has allowed certain reference laboratories and in-clinic tabletop analyzers to offer routine measurement of [SDMA].

B. Results from different assays and analyzers can vary, thus appropriate laboratory-specific RIs and decision limits should be used when available. A point-of-care analyzer had a bias compared to the same company's commercial laboratory immunoassay; including the assays' imprecision, errors in that study exceeded performance goals for an SDMA assay.[90]

C. Sample: Serum or heparinized plasma are used, but it is not clear if they yield the same results.[90] Direct comparisons were not found. Effects of freezing are not clear.[90] Based on proprietary data, IDEXX has recommended that kittens < 6 mo of age not be sedated prior to venipuncture because [SDMA] may be falsely increased.

D. Unit conversion: 20.2 µg/dL = 1 µmol/L (SI unit)

III. Interpretation of increased [SDMA]
   A. The biological variation of [SDMA] in cats appears to be intermediate, so it may be valuable to use subject-based changes as well as comparison to population-based RIs for interpretation of [SDMA].[91]
   B. Decreased GFR: As with any method of detecting decreased GFR, disorders that reduce renal blood flow (e.g., hypovolemia, dehydration, decreased cardiac output, vasoconstriction resulting from urinary tract obstruction) can cause a decreased GFR and thus potentially an increased [SDMA]. Increased plasma [SDMA] in dogs, cats, and horses occurs with prerenal, acute and chronic renal, and postrenal causes of azotemia; it is not specific for renal disease.
      1. Studies in cats with chronic renal disease have shown that there is a positive correlation (R^2 = 0.55) between serum [SDMA] and serum [Crt].[92] When assessed by iohexol plasma clearance, GFR was reciprocally related to plasma [Crt] (R^2 = –0.81[93] or –0.59[94]) and to serum [SDMA] (R^2 = –0.82[93] or –0.62[94]). In 21 cats with chronic renal disease, serum [SDMA] reportedly increased months sooner than serum [Crt] (the authors used a [Crt] upper reference limit of 2.1 mg/dL, but the highest value in the 21 healthy cats of the study was only 1.6 mg/dL; if a lower [Crt] decision limit was more appropriate for this population, [Crt] would have been increased sooner in more cats with chronic renal disease).[94]
      2. Studies in dogs with chronic renal disease have shown that SDMA concentrations increase as GFR decreases.[95] [SDMA] increased over its upper reference limit in 17 of 19 dogs with chronic renal disease from 2–27 mo before serum [Crt] increased above its upper reference limit.[96]
      3. In addition to detecting decreased GFR sooner than serum [Crt], there is evidence that [SDMA] in dogs is less affected than [Crt] by variations in body weight (see Creatinine (Crt) Concentration in Serum or Plasma, Section III). In 38 clinically healthy beagles > 3 yr of age, [SDMA] was not influenced by differences in lean body mass of 5–13 kg in dogs weighing 9–18 kg at the start of the study, whereas there was a modest correlation between lean body mass and serum [Crt] (r = 0.54).[56] For unexplained reasons, [SDMA] decreased over the 6-mo duration of the study;[56] the mean ± SD values for [SDMA] at baseline, 1 mo, 3 mo, and 6 mo were 13 ± 5, 13 ± 3, 8 ± 2, and 6 ± 2 µg/dL, respectively. The authors stated their RI was < 14 µg/dL, thus some dogs had SDMA concentrations above the RI early in the study. The analyzed samples had been frozen at –70 °C (duration not stated).
      4. Effects of hyperthyroidism and hypothyroidism on [SDMA] are not clear, but caution in interpretating SDMA results in the presence of thyroid disease is warranted.[45,97]
   C. Increased SDMA production
      1. Neoplasms: Dogs and cats with lymphoma have a greater probability of increased serum [SDMA], often without increased [Crt], compared to dogs and cats with other neoplasms.[98] Neoplastic cells may overexpress protein arginine methyltransferase 5 and thus produce and release increased amounts of SDMA.
      2. Proteolysis: In an experimental model of acute renal insufficiency in rats, the increased SDMA concentrations were reportedly the result of increased SDMA formation and not decreased urinary excretion. The authors concluded that metabolic acidosis of acute renal insufficiency may have caused increased proteolysis and increased SDMA production. The experimentally induced acute renal disease decreased GFR, but urinary excretion of SDMA was maintained by decreased tubular resorption of SDMA.[99]

ABNORMAL ROUTINE SERUM OR PLASMA CHEMISTRY RESULTS IN AZOTEMIC ANIMALS

I. [Inorganic phosphorus]
   A. In dogs, cats, horses, and camelids,[100] hyperphosphatemia occurs when renal clearance of plasma PO_4 is substantially reduced due to decreased GFR caused by renal disease (acute or chronic) or other pathologic states (e.g., prerenal or postrenal azotemic states).
   B. Some azotemic horses have a low-normal [inorganic phosphorus] or hypophosphatemia due to decreased renal conservation of PO_4 or to extrarenal excretion of PO_4.
   C. Azotemic cattle may or may not be hyperphosphatemic. Renal excretion of PO_4 is minor compared to excretion via saliva and the rumen.[38,101]

II. [tCa^{2+}]
   A. The [tCa^{2+}] may be below, above, or within RIs in all species.
   B. Dogs, cats, and cattle usually have a low-normal [tCa^{2+}] or mild hypocalcemia in chronic renal failure because of decreased renal functional mass and therefore decreased renal formation of $1,25(OH)_2D$. Hypercalcemia occurs in a minority of cases. In cattle, PO_4 excreted in saliva may bind with Ca^{2+} in the alimentary tract and make it less available for absorption.[38]
   C. In dogs with chronic renal failure, the [tCa^{2+}] may not reflect the regulation of the [fCa^{2+}] because the amount of Ca^{2+} bound to nonprotein anions may be increased (see Chapter 11).
   D. If their diet is relatively rich in Ca^{2+}, horses are often hypercalcemic in acute or chronic renal disease because of decreased renal clearance of plasma Ca^{2+}; a major renal function of horses is to excrete excess dietary Ca^{2+}. Depending on the cause of the renal disease and the dietary intake of Ca^{2+}, horses can be hypocalcemic during renal failure.
   E. In alpacas and llamas, the changes in plasma concentrations of Ca^{2+} and PO_4 are dependent on diet and vitamin D status.[14]

III. [K^+] and blood pH ([H^+]) (see Chapters 9 and 10)
   A. Dogs, cats, and horses
      1. Acute decreases in renal function may cause hyperkalemia and acidemia because of impaired renal excretion of K^+ and H^+. Also, inorganic acidemia accentuates hyperkalemia through the redistribution of ions between intracellular and extracellular compartments (Figure 9.5).
      2. Hyperkalemia and acidemia occur mostly with oliguric or anuric renal failure and can occur with either acute renal failure or terminal chronic renal failure.
      3. Many other factors influence serum [K^+] and blood pH, so expected changes may not occur.
   B. Cattle[102,103]
      1. Cattle with renal azotemia tend to be hypokalemic due to alkalemia, decreased dietary potassium intake, or increased potassium excretion via saliva.[38]
      2. The animal's metabolic alkalosis and concurrent hypochloremia are often considered the result of H^+ and Cl^- sequestration in the abomasum due to abomasal atony. However, the metabolic alkalosis (i.e., ↑ [HCO_3^-]) is more accurately attributable mostly to excessive formation of HCO_3^- by the abomasal mucosa and renal tubules. The formation of HCO_3^- is explained in Chapter 9 (Figure 9.6).
      3. Cattle tend to have concurrent hyponatremia, suggesting loss of Na^+ and Cl^-.

C. Alpacas and llamas
   1. Urinary tract rupture in alpacas and llamas reportedly tends to cause metabolic alkalosis (not explained), hyponatremia, hypochloremia, and hypermagnesemia with varied changes in [K^+] attributed to varying degrees of decreased dietary K^+ intake that may limit increases expected with impaired renal K^+ excretion.[14]
   2. Metabolic acidosis but otherwise similar changes reportedly occur with acute toxic nephrosis, again with K^+ changes varying from hyperkalemia when renal dysfunction is severe to hypokalemia with lower grade disease and decreased K^+ intake.

IV. α-Amylase and lipase activities (see Chapter 12)
   A. Dogs: Canine kidneys provide a major route of α-amylase and lipase excretion or inactivation. If renal perfusion or functional renal mass is decreased, there is less renal excretion or inactivation of α-amylase and lipases, and thus their long half-lives are increased. With time, mildly to moderately increased serum α-amylase and lipase activities may develop.
   B. Cats: The involvement of feline kidneys in the clearance or inactivation of α-amylase and lipase is not well documented. Of 32 cats with renal failure, hyperamylasemia (slight to 3× increase) was present in 10.[104]

MAJOR URINALYSIS (UA) CONCEPTS

I. Components of a routine UA may vary from one laboratory to another; however, all procedures are best done on fresh urine (< 1 h and no more than 4 h old). Besides the potential for deterioration of cells and casts, delay of 6–24 h in completing the UA may allow in vitro crystal formation (especially if the sample is stored at 4 °C), crystal dissolution, bacterial proliferation, and loss of volatile compounds.[105]

II. Analytical and quality assurance procedures for a routine UA have been described,[106–109] and automated methods are discussed later in this chapter.

III. Common components of most routine urinalyses
   A. Physical examination: color, clarity, and USG_{ref}
   B. Chemical examination by reagent-strip methods
      1. Common assays: pH, protein, glucose, ketone, heme (occult blood), bilirubin, and urobilinogen
      2. Other assays: nitrite, USG, and leukocyte esterase
   C. Sediment examination: microscopic examination to identify RBCs, WBCs, bacteria, casts, crystals, epithelial cells, and other nondissolved material (may be done with nonstained or with stained urine sediment preparations)

IV. Urine composition is determined by three major factors. Because of these factors, the urine composition is affected by the entire urinary system and by other body systems.
   A. Quantity and composition of the plasma presented to kidneys
   B. Renal functions, including filtration, tubular secretion, and resorption
   C. Material (chemicals and cells) added to the glomerular filtrate as it flows through kidneys, ureters, urinary bladder, urethra, and prepuce or vagina and vulva

V. It is important to know the method of urine collection (i.e., voided, cystocentesis, catheterization, or "off-surface") when the UA results are being interpreted. Voided samples may have more bacteria, epithelial cells, and WBCs from the distal urethra and genital tract. Cystocentesis samples may be affected by iatrogenic hemorrhage, but they localize abnormalities to anywhere from the kidneys to the proximal urethra. Catheterized samples may have more epithelial cells, blood from iatrogenic hemorrhage, lubricant, and bacteria, and "off-surface" samples may be contaminated with a variety of microscopic particulates. Results expected in healthy dogs, cats, horses, cattle, and camelids are listed in Table 8.3. These expected findings for healthy animals are not quite analogous to typical RIs; although they are the usual findings, healthy animals in different hydration states may have different findings. UA results must be interpreted relative to hydration status.

PHYSICAL EXAMINATION OF URINE

I. Urine color (pigments)
   A. Physiologic processes
      1. The normal yellow to amber color is due to *urochromes*, a group of poorly defined urine pigments of which one is riboflavin.
      2. Pale yellow urine is usually less concentrated than dark yellow urine, but not always (Figure 8.8).
   B. Analytical concepts: Gross assessment of urine color is typically done on fresh, well-mixed urine.
   C. Abnormal urine color (*pigmenturia*)
      1. An abnormal color indicates the presence of abnormal pigments in the urine. Other parts of the UA or other assays are needed to determine which pigment or pigments are present.
      2. Although concurrent pigmenturia may alter expected colors, the following are common abnormal colors and the substances that create them:
         a. Red: RBCs, hemoglobin, or myoglobin
         b. Red-brown: RBCs, hemoglobin, myoglobin, or methemoglobin
         c. Brown to black: methemoglobin from hemoglobin or myoglobin
         d. Yellow-orange: bilirubin
         e. Yellow-green or yellow-brown: bilirubin or biliverdin
      3. Horse urine may turn red or brown during storage or when exposed to snow. The pigmenturia is reported to be caused by pyrocatechin (pyrocatechol),[110] which is the

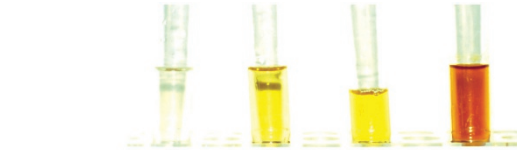


Figure 8.8. Urine appearance. Photograph of urine samples with different gross colors showing that appearance does not necessarily predict USG_{ref} or solute concentration (see tabular data), but it usually does.

aromatic portion of catecholamines. A pathologic state is not associated with this pigmenturia.

II. Urine clarity
- A. Physiologic processes
  1. Clear urine is expected in most species, but it may have mild turbidity due to suspended particles (e.g., epithelial cells and crystals).
  2. Equine urine is frequently turbid or cloudy because of the presence of mucoprotein (produced by kidneys) or calcium carbonate crystals.
- B. Analytical concepts: Gross assessment of urine clarity is typically done on fresh, well-mixed urine. It may be simply described or graded by the degree to which printed material cannot be read clearly through a tube of the urine.
- C. Cloudiness or turbidity indicates the presence of formed elements, such as cells, crystals, bacteria, casts, and lipid droplets in the urine. Protein does not cause turbidity.

III. Solute concentration
- A. Physiologic processes
  1. The solutes in urine are the dissolved ions and molecules. Most of them, including electrolytes (Na^+, K^+, Cl^-, Ca^{2+}, PO_4, and NH_4^+) and additional metabolic products (urea and Crt), are being excreted by the kidneys.
  2. The concentrations of filtrate solutes are modified by the tubular resorption or secretion of solutes and by the resorption of filtrate H_2O.
  3. Urine solute concentrations are expected to increase when the kidneys are conserving H_2O and decrease when the kidneys are not conserving H_2O. Therefore, USG varies over time unless renal function is sufficiently impaired.
  4. Healthy neonatal foals produce hyposthenuric urine;[111] they may be in a state of diuresis for several months while nursing.
- B. Analytical concepts
  1. *Specific gravity* (also called *relative density*), a physical property of a solution, is the ratio of a solution's weight to the weight of an equal volume of H_2O (i.e., the ratio of their densities). Measurement of the true urine SG is an obsolete procedure in clinical medicine. Specific gravity is a unitless ratio.
  2. Refractive index as an estimate of USG (USG_{ref})
     - a. The refractive index of urine is measured with a refractometer and used as a routine clinical estimation of USG (USG_{ref}). The *refractive index* (*n*) of a solution is the ratio of the speed of light in a vacuum to the speed of light in the solution. When light waves enter a solution, they slow down and bend (refract). As solute is added to H_2O, the degree to which the light slows and is refracted increases proportionately to the increase in solute concentration (i.e., the refractive index increases). Specific gravity also increases proportionately to the solute concentration, so specific gravity correlates with refractive index if the types and proportions of the solutes remain similar. If urine has relatively normal solute composition, USG_{ref} from a good-quality refractometer correlates very well with osmolality.
     - b. Optical refractometers have been used for many decades to estimate USG. Using the same physical properties, digital refractometers have also been developed for estimating USG. Studies have shown that the results of calibrated and well-maintained optical and digital refractometers provide similar results (e.g., ± 0.002).[112–114] Interobserver variability should be clinically insignificant when users

are trained and optical or digital refractometers are well maintained.[115] As with all refractometers, different results are obtained if there are different methods of converting a urine's refractive index to a specific gravity value.

c. The refractive index of a solution is highly dependent on three factors: (i) solute concentration, (ii) chemical composition of the solutes, and (iii) temperature. At a given temperature and with a given composition, a fluid's refractive index and therefore USG_{ref} increases as total solute concentration increases. To understand this, consider the following.
   (1) *Refraction* of a fluid is defined as the difference in refractive index between that fluid and its solvent, typically written as the difference times 10^4 to minimize decimals (Eq. 8.2a).
   (2) *Refractivity* has been defined as the ratio of refraction to solute concentration (Eq. 8.2b), a value that remains fairly constant over wide ranges of concentrations for a particular solute.[116]
   (3) Different solutes have different refractivity values. Those that cause more refraction at a given concentration have greater refractivity. Even though the composition of urine is complex, the types of solutes in the urine tend to be similar and thus the combined refractivity of those solutes tends to be similar. The refractivity of human urine is 16 whereas the refractivity of NaCl is 17, that of urea is 15, and that of glucose is 14.[116] If the refractivity is nearly constant in urine samples, then the refraction is proportional to TS % (Eq. 8.2c).
   (4) The TS % is defined as the percentage of total weight of the solution that is due to solids (see Eq. 8.2d). Eq. 8.2d is created by substituting the TS % formula into Eq. 8.2c. If Eq. 8.2d is compared to the formula for specific gravity (Eq. 8.2e), one can see the similarities of refraction and specific gravity: both contain a weight of the solution (one in the denominator, one in the numerator), and both depend on the ratio of two weights. It is important to remember that the USG_{ref} is not truly a specific gravity value – it is an estimate that is dependent on the conversion of refraction to a specific gravity value.
   (5) The similar aspects of a solution's refraction and its specific gravity allow us to use the refractometer to estimate the specific gravity of urine. This estimate is generally a reliable predictor of a solution's osmolality, as long as there is not abnormal solute that greatly increases the solution's refraction (see III.B.5 in this section and Figure 8.11). In this context, glucose and protein are abnormal solutes for urine and they can contribute significantly to the solution's refraction, but their contributions to urine osmolality are minimal.
   (6) The temperature-dependent factors of the refractive index are minimal if a temperature-compensated refractometer is used.

$$\text{refraction} = (n - n_0) \times 10^4 \quad (\mathbf{8.2a})$$

n = refractive index of solution and n_0 = refractive index of H_2O

Example: refraction = $(1.3442 - 1.3330) \times 10^4 = 0.0112 \times 10^4 = 112$

$$\text{refractivity of solute} = \frac{\text{refraction}}{\text{dissolved solute percentage}} = \frac{\text{refraction}}{\text{TS}\,\%} \quad (\mathbf{8.2b})$$

$$\text{(rearranging 8.2b) refraction} = \text{refractivity of solutes} \times \text{TS}\,\% \quad (\mathbf{8.2c})$$

$$\text{Because TS \%} = \frac{\text{g of solute}}{100\text{ g of solution}}\text{, Eq. 8.2c becomes:}$$

$$\text{refraction} = \text{refractivity of solutes} \times \frac{\text{g of solute}}{100\text{ g of solution}} \quad (\mathbf{8.2d})$$

$$\text{specific gravity} = \frac{\text{weight of solution}}{\text{weight of } H_2O} \text{ (with equal volumes of solution and } H_2O) \quad (\mathbf{8.2e})$$

d. USG_{ref} may be measured before or after urine centrifugation, i.e., for whole urine or urine supernatant. Refractometers measure the refractive index of the soluble solids in the fluid (i.e., solutes in the solvent); suspended particles (e.g., cells, casts, and most crystals) do not alter the refractive index of urine.[117] Because the suspended particles interfere with light transmission (thereby causing cloudiness or turbidity), they may make the demarcation line in the refractometer more difficult to read. The following are USG_{ref} values for 10 cloudy urine samples (direct reading/supernatant reading): 1.043/1.043, 1.054/1.054, 1.028/1.028, 1.016/1.016, 1.083/1.083, 1.021/1.022, 1.051/1.052, 1.048/1.048, 1.061/1.062, and 1.051/1.052 (authors' unpublished data).

e. Most temperature-compensated refractometers have USG_{ref} scales that are calibrated for the composition of urine from healthy people (Figure 8.9).

f. However, there are refractometers that have different USG_{ref} scales for feline as compared to canine and large animal urine samples (Figure 8.10).
   (1) The scale for dogs and large animals is slightly different from the calibration scale for people.
   (2) The scale for cats is somewhat different: 1.010 on the canine scale is about 1.008 on the feline scale, 1.025 on the canine scale is about 1.022 on the feline scale, and 1.040 on the canine scale is about 1.036 on the feline scale. In a study in which refractometric values from a feline scale were compared with values from a pycnometer, the USG_{ref} values from the feline scale were 0.007 to 0.008 lower and thus may not be accurate.[118] Note: A pycnometer is a glass flask that allows precise measurement of fluid volumes for comparison of the weights of equal volumes of fluids; the ratio of the weight of a volume of urine to the weight of an equal volume of H_2O yields the urine's specific gravity.

g. If refractive indices of human and feline urine were 1.3365, the USG_{ref} would be 1.010 and 1.008, respectively. If the refractive indices were 1.3420, the USG_{ref} would be 1.025 and 1.021, respectively.[116] If a refractometer calibrated for human urine is used for cat urine, the urine appears slightly more concentrated than it really is.

h. Refractometers that are not temperature compensated underestimate the USG when ambient temperature increases above 68 °F (20 °C). The amount of error increases as the temperature increases but for most indoor clinical environments, the error is not clinically significant.

i. When the USG_{ref} is greater than the upper end of the USG scale of the refractometer, a direct reading cannot be obtained. If a reading can be made on the refractive index scale, some labs convert the refractive index value to a USG based on tables generated experimentally. If the reading is also greater than the refractive index scale, the sample may be diluted (equal volumes of urine and distilled H_2O),

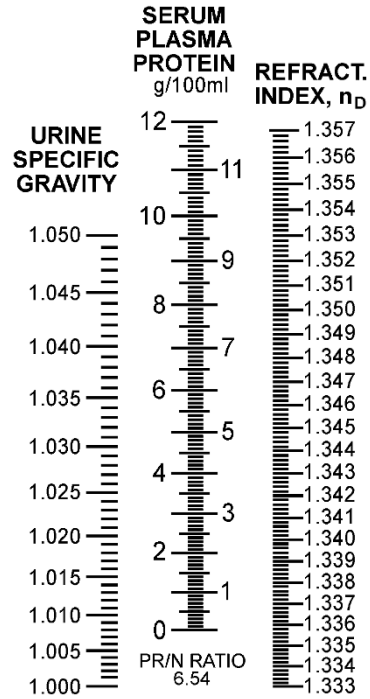


Figure 8.9. Illustration of scales in a Leica TS400 hand-held refractometer. The refractometer measures the fluid's refractive index, and the *urine specific gravity* and *serum plasma protein* scales are used to estimate USG_{ref} and serum or plasma total protein concentrations, respectively. The refractometer scales were calibrated for human samples. PR/N ratio 6.54 is a conversion factor based on each 6.54 g of protein containing 1 g of nitrogen. Image used with permission from Leica Microsystems, Buffalo, NY.

reevaluated, and reported after correcting for the dilution (a 1.026 result with the diluted sample would be reported as 1.052). Such a calculated USG value is only an approximate value because each unit of change in refractive index does not directly translate to a unit of change in the USG scale (the relationship is nonlinear). From Wolf's tables for human urine, when USG differs by 0.015 when going from 1.000 to 1.015, the refractive index changes by 0.0053. However, when the USG differs by 0.015 when going from 1.015 to 1.030, the refractive index changes by 0.0059.[116] Diagnostically, it is usually not necessary to know how concentrated the urine is when it is too concentrated to be read directly from the refractometer (i.e., $USG_{ref} > 1.050$).

3. Osmolality (see Chapter 9 for interpretation of serum osmolality values)
   a. Principle (*freezing-point osmometry* method): The freezing point of a solution is inversely related to the solute concentration in the solution. As the solute concentration increases, the freezing point decreases (putting salt on ice lowers the freezing point so ice may melt at the same temperature). A freezing-point osmometer detects the freezing point of a solution and converts the value to osmolality.
   b. *Osmolality*, which is the solute concentration of a solution, can be expressed in osmoles of solute particles per kilogram of solvent (osmol/kg) or in moles of solute

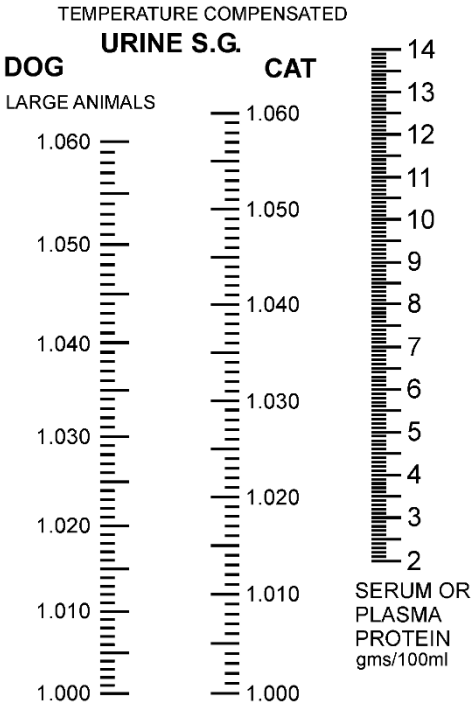


Figure 8.10. Illustration of scales in a Leica VET 360 hand-held veterinary refractometer. The refractometer has a specific gravity scale for dog and large animal urine and a separate scale for cat urine. Note that, for a given refractive index, the specific gravity indicated for cat urine is less than the specific gravity for dog or large animal urine. However, the accuracy of a separate scale has been questioned (see text). Image used with permission from Leica Microsystems, Buffalo, NY.

per kilogram of solvent (mol/kg); 1 osmol is 1 mol of osmotically active particles. For a substance that does not dissociate in solution, 1 mol = 1 osmol. For a substance that completely dissociates into two ions per mole, each 1 mol of the substance generates 2 osmol and 2 mol of particles.

c. Urine osmolality measurements are usually limited to specific cases where accurate assessment of renal concentrating or diluting abilities is critical. The gold standard for assessing urine solute concentration is freezing-point osmometry.

4. USG_{ref} as an estimate of osmolality

a. Figure 8.11 illustrates the relationship between USG_{ref} and urine osmolality measurements. In most urine samples, there is a good linear relationship between USG_{ref} and osmolality. Thus, the USG_{ref} is typically a good reflection of the urine solute concentration. However, it provides only an approximate value, as samples with identical osmolalities in Figure 8.11 were associated with a range of USG_{ref} values: 8 samples with a USG_{ref} of 1.024 had urine osmolalities from 833 to 972 mmol/kg H_2O; 10 samples with a USG_{ref} of 1.020 had urine osmolalities from 454 to 738 mmol/kg H_2O; 12 samples with a USG_{ref} of 1.015 had urine osmolalities from 392 to 643 mmol/kg; 11 samples with a USG_{ref} of 1.010 had urine osmolalities from 278 to 435 mmol/kg H_2O.

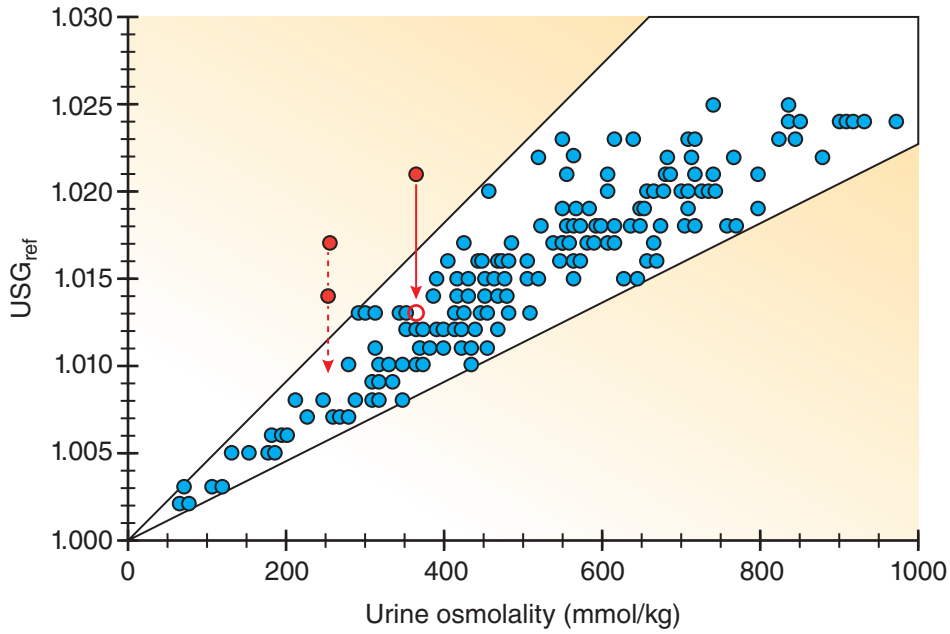


Figure 8.11. Comparison of osmolality and USG_{ref} in canine urine samples with $USG_{ref} \leq 1.025$ (n = 185). There is a high correlation (r = 0.91) between the two methods of assessing urine solute concentration: freezing-point osmometry and refractive index. For these samples, the mean Osm_u:USG_{ref} was 33 with a SD of 5; the mean ± 2 SD (95.45 % interval) is represented by the *white area between the two diagonal lines*. The three values (*red dots*) that fell above this interval had high protein concentrations (estimated concentrations of 500–2000 mg/dL). If the greatest reading (1.021) were corrected for a protein concentration of 2 g/dL by subtracting 0.004 for each 1 g/dL of protein, the USG_{ref} would be 1.013 (*red open circle*). The USG_{ref} values for the other red dot samples would most likely correct to values within the diagonal lines.

b. Because of this good correlation, the Osm_u:USG_{ref} ratio is relatively constant (Eq. 8.3a). In 185 canine urine samples with $USG_{ref} < 1.025$, the Osm_u:USG_{ref} ratio was 33 ± 5 (mean ± SD). In 37 feline urine samples with $USG_{ref} < 1.025$ (using nonfeline scale), the Osm_u:USG_{ref} ratio was 35 ± 5 (mean ± SD) (SLS, unpublished results). The ratios can be used to estimate the urine osmolality from the USG_{ref} so that it can be compared to a patient's serum osmolality (Eq. 8.3b).

$$(\text{Osm:USG}_{\text{ref}})_u = \frac{\text{Urine osmolality}}{1000 \times (\text{USG}_{\text{ref}} - 1.000)}$$

Example: If urine osmolality = 600 mmol/kg and USG_{ref} = 1.020, then: **(8.3a)**

$$(\text{Osm:USG}_{\text{ref}})_u = \frac{600}{1000 \times (1.020 - 1.000)} = \frac{600}{1000 \times (0.020)} = \frac{600}{20} = 30$$

OR

$$(\text{Osm:USG}_{\text{ref}})_u = \frac{\text{Urine osmolality}}{\text{Last 2 digits of USG}}; (\text{Osm:USG}_{\text{ref}})_u = \frac{600}{20} = 30$$

Estimated canine serum osmolality = last 2 digits of USG_{ref} × (33 ± 10)
Example: If USG_{ref} = 1.025, estimated canine serum osmolality = 25 × (33 ± 10) = 825 ± 250 mmol/kg (**8.3b**)

c. When interpreting either the USG_{ref} value or urine osmolality, it may be important to recognize the following relationships:
   (1) If urine 1 has an osmolality of 300 mmol/kg and urine 2 has an osmolality of 600 mmol/kg, the solute concentration in urine 2 is twice the solute concentration in urine 1.
   (2) If the USG_{ref} of urine 3 is 1.010 and the USG_{ref} of urine 4 is 1.020, the solute concentration in urine 4 is approximately twice the solute concentration in urine 3.

5. Effect of marked proteinuria or glucosuria on USG_{ref}: When there is either marked proteinuria or marked glucosuria, the USG_{ref} overestimates the concentration of urine solutes and thus renal concentrating ability (Figure 8.11). The Osm_u:USG_{ref} ratio will be decreased (in dogs, below 23). Common guidelines for the effect are as follows:
   a. A [protein] of 1 g/dL adds about 0.003–0.005 to USG_{ref} but has almost no effect on osmolality. The effect of milder proteinuria is proportionately less and generally insignificant.
   b. A [glucose] of 1 g/dL adds about 0.004–0.005 to USG_{ref} and only slightly increases urine osmolality (~5 mmol/kg H_2O). The effect of milder glucosuria is proportional and generally insignificant. The interpretation of one published study was that "glucosuria in samples from dogs and cats did not prevent accurate assessment of urine concentrating ability,"[119] but consideration should be given to the following:
      (1) In that study, addition of glucose to urine samples with USG_{ref} in the isosthenuric range caused USG_{ref} increases similar to the magnitude noted above. Such increases could alter recognition of hyposthenuria and isosthenuria.
      (2) High-glucose samples (2.4 g/dL) were reportedly made by adding a stock solution of glucose in H_2O (10 g/dL), thus causing a 24 % dilution of urine with H_2O while adding glucose. The H_2O dilution would have decreased the USG_{ref} of a sample from 1.040 to about 1.030, while the glucose would have increased the USG_{ref} by about 0.010–0.012, essentially canceling out and therefore not revealing a glucose effect at high [glucose]. In contrast, the added H_2O would have caused a decrease of only about 0.002 in a "waterier" urine sample with USG_{ref} = 1.010, but the added glucose would have increased the USG_{ref} by about 0.010–0.012, yielding the reported net increases.
   c. If the maximum protein and glucose reactions on a urine reagent strip reflect concentrations of ≥ 2 g/dL, then a maximal reading for urine [protein] or [glucose] would indicate that the sample's USG_{ref} is falsely increased by an increment of ≥ 0.006 or 0.008, respectively.

6. The following data illustrate why the presence of glucose or protein can cause a significant increase in USG_{ref} whereas they cause very little change in urine osmolarity or osmolality. In actual urine samples, the effects of glucose and proteins differ slightly

because glucose and proteins found in serum have different refractivity values: 19 for serum proteins, 14 for glucose.[116]

a. Considering the typical composition of human urine in health, a urine's TS % is 2.2 g/100 g at a specific gravity of 1.010 (refractive index 1.3365).[116] If enough glucose were added to urine to raise the urine glucose concentration to 1 g/dL, then the urine's TS % would be near 3.2 g/100 g and thus its refractive index would be 1.3380 and its USG_{ref} would be 1.014 (Figure 8.9, right and left scales). The greater glucose concentration would raise the urine's osmolarity ~6 mmol/L, i.e., from ~300 mmol/L to ~306 mmol/L. Thus, the glucose concentration of 1 g/dL raises the USG_{ref} by about 0.004 whereas it has negligible effect on urine osmolarity. If enough glucose were added to create a glucose concentration of 2 g/dL, the urine's refractive index would increase the USG_{ref} by about 0.008, to 1.018, but the urine osmolarity would increase to only 312 mmol/L.

b. The same type of calculations can be made for the addition of protein to create a urine protein concentration of 1 g/dL. Again, the protein would increase the USG_{ref} by about 0.004 but would have essentially no effect on urine osmolarity because the osmolarity of a protein concentration of 1 g/dL is much less than 1 mmol/L. The positive protein interference is illustrated in Figure 8.11.

7. Mild to marked increases in apparent USG_{ref} (to > 1.100) may be caused by renal excretion of contrast material (e.g., iohexol) into urine formed within a few hours after a contrast study.[120] Such USG_{ref} values do not reflect osmolality or renal concentrating ability.
8. Reagent strip for estimating USG (Siemens)
   a. The reagent-strip method is not recommended for estimation of urine solute concentration or USG of domestic mammals.
   b. Principle: The ionic strength of urine is related to total solute concentration. The reagent system has indicators that produce different colors when dipped in urine samples of different ionic strengths.
   c. The semiquantitative scale is 1.000–1.030 at 0.005 intervals, with best accuracy when the urine pH is < 6.5. Add 0.005 to the reading for a pH ≥ 6.5 (Siemens Multistix package insert).
   d. Falsely low results occur in alkaline urine; most urine samples with a pH ≥ 7.0 and a USG_{ref} of 1.025–1.035 had a dipstick USG of 1.015.[121] Moderate quantities of protein can cause falsely high values, and glucosuria can cause falsely low values.[122]

C. Expected USG_{ref}
   1. One must have knowledge of an animal's H_2O balance and medications to properly assess an animal's ability to concentrate or dilute urine.
      a. An animal that is dehydrated or has restricted access to H_2O should excrete a relatively small volume of concentrated urine, i.e., with a relatively high USG_{ref}.
      b. After diuretic or fluid therapy, an animal should excrete a relatively high volume of less-concentrated, isosthenuric, or dilute urine, i.e., urine with a lower USG_{ref}.
   2. Healthy animals can excrete urine with a broad USG_{ref} range (Table 8.3), depending on what the kidneys are being challenged to do.
      a. Maximal urine dilution in domestic mammals as assessed by USG_{ref} is near 1.001.
      b. Maximal urine concentration as assessed by USG_{ref} is > 1.080 for cats, near 1.060 for dogs, and near 1.050 for horses, cattle, alpacas, and llamas.

D. Interpretation of USG_{ref} values

1. A USG_{ref} is usually needed for the assessment of renal concentrating ability when animals are azotemic, polyuric, oliguric, or anuric. Because of the many variables that change urine solute concentrations, it is difficult to formulate firm guidelines for the interpretation of USG_{ref} in all cases.
2. Animals with impaired renal concentrating ability have one or more of the following defects (mechanisms for the resulting polyuric states are listed in Table 8.10):
   a. ADH deficiency is present (central diabetes insipidus).
   b. Epithelial cells of collecting ducts are not responsive to ADH (nephrogenic diabetes insipidus).
   c. Solute overload was present (too much solute entering the LoH as occurs with osmotic diuresis, renal failure, or increased GFR), causing a high flow rate and decreased resorption of tubular H_2O.
   d. Decreased medullary hypertonicity

Table 8.10. Major pathogenic mechanisms of polyuria

	Solute diuresis	↓ tubular response to ADH	↓ medullary tonicity	↓ ADH
Chronic renal failure	+[a]	+	+	–[b]
Acute renal failure	–	+[c]	–	–
Postobstructive diuresis[d]	+	+[c]	–	–
Diabetes mellitus	+	–	–[e]	–
Hypercalcemia	–	+	–[e]	–
Canine pyometra	–	+	–[e]	–
Hypokalemia	–	+	–[e]	–
Hyperthyroidism	+	+	–	–
Hypoadrenocorticism	–	–	+[f]	±[g]
Hepatic failure	–	±[h]	+	–
Hypothalamic or pituitary disease	–	–	–[e]	+
Hyperadrenocorticism	–	–[i]	–[e]	+[i]
Psychogenic polydipsia	–	–	–[e]	+[j]

[a] A *plus sign* indicates that mechanism can contribute to the polyuria.
[b] A *minus sign* indicates that mechanism does not contribute to the polyuria.
[c] Initially, there may be oliguria. If the animal survives the acute illness, the surviving but damaged tubules may not be able to respond to ADH adequately.
[d] Altered Na^+-regulatory hormone responses and other defective tubular functions may contribute.
[e] Persistent diuresis may decrease medullary tonicity because of decreased tubular resorption of solutes.
[f] Persistent hyponatremia and hypochloremia may result in LoH failure.
[g] Hypoosmolality would decrease the stimulus for ADH release, but concurrent hypovolemia would stimulate ADH release.
[h] An increased NH_4^+ concentration may interfere with tubular response to ADH.
[i] See text for explanation.
[j] An increased GFR may also contribute.

(1) Prolonged hyponatremia or hypochloremia
(2) Defective Na^+ and Cl^- transport in the LoH (e.g., loop diuretics)
(3) Decreased urea production because of hepatic disease
(4) Solute overload or prolonged diuresis

3. A pathologic state or another condition that results in a lower medullary osmolality reduces the ability of the kidneys to concentrate urine. This state is referred to as *medullary washout* when the decreased medullary tonicity is caused by a high flow rate of fluid through renal tubules and consequently less resorption of the major medullary solutes (i.e., Na^+, Cl^-, and urea); the solutes are "washed out."
4. General guidelines for USG_{ref} interpretation are in Table 8.11.
5. USG_{ref} in various disorders or conditions (Note: The provided USG_{ref} values should be used as guidelines, not firm decision thresholds. Data are lacking for alpacas and llamas; information for horses and cattle might apply.)[118]
   a. USG_{ref} > 1.030 in an oliguric dog, > 1.040 in an oliguric cat, and > 1.025 in oliguric horses or cattle
      (1) Nonrenal processes (e.g., hypovolemia and decreased cardiac output) have led to decreased renal perfusion. Hypovolemia or plasma hyperosmolality stimulated the release of ADH.
      (2) ADH promoted the resorption of H_2O in the collecting ducts, thus concentrating the tubular fluid and thus the urine.
   b. USG_{ref} < 1.030 in an obviously dehydrated dog, < 1.040 in a dehydrated cat, and < 1.025 in a dehydrated cow or horse
      (1) Such findings indicate a renal concentrating defect that could be caused by renal or extrarenal disease.
      (2) Pathogeneses of the defects vary with the pathologic states (see the polyuric disorders in the following Sections c and e–g).
   c. USG_{ref} = 1.020–1.035 in a polyuric animal
      (1) Diabetes mellitus: Glucosuria causes osmotic diuresis by inhibiting the passive resorption of H_2O in the PCTs. Also, if it persists, medullary tonicity may decrease because a high tubular flow rate impairs resorption of Na^+, Cl^-, and urea.
      (2) Potentially seen with renal glucosuria
      (3) Partial diabetes insipidus or hypoadrenocorticism: An animal may be polyuric with a corresponding low USG_{ref} on some days, but have values in the 1.020–1.035 interval on other days.
      (4) Renal failure: Concentrating ability may be incompletely impaired when kidneys are failing, but a lower USG_{ref} is more typical of polyuric renal failure.
   d. USG_{ref} = 1.007–1.013 in an oliguric animal
      (1) This typically indicates renal failure or end-stage renal disease if the animal is azotemic, but it could represent acute renal disease or urinary tract obstruction.
      (2) Concentrating and diluting defects could be caused by solute diuresis, decreased tubular response to ADH, or impaired ability to maintain a medullary solute gradient.
   e. USG_{ref} = 1.007–1.013 in a polyuric animal
      (1) If there is concurrent azotemia, such findings are essentially diagnostic of renal failure.
      (2) Concentrating and diluting defects are caused by solute diuresis, decreased tubular response to ADH, or impaired ability to maintain a medullary solute gradient.

Table 8.11. Guidelines[a] for interpretation of USG_{ref} values in dogs

Case information	USG_{ref}[b]	Interpretation
Nothing known	1.001–1.060	Could be found in a clinically healthy or sick dog
Dehydrated	> 1.030	Reflects renal attempts to conserve H_2O appropriately
	1.014–1.030	Suggests impaired renal concentrating ability and possibly renal failure; could be seen with glucosuria, hyponatremia and hypochloremia, partial renal diabetes insipidus disorders, hypoadrenocorticism
	1.007–1.013	Indicates defective renal concentrating ability; if azotemic, then renal insufficiency or failure until proven otherwise
	< 1.007	Indicates defective renal concentrating ability but not due to renal failure, as kidneys have ability to dilute ultrafiltrate; consider central or renal diabetes insipidus
Polyuria	> 1.020	Reflects renal attempts to conserve H_2O and thus probably not in renal failure; could be seen with glucosuria, hyponatremia and hypochloremia, or partial central or renal diabetes insipidus disorders
	1.007–1.013	Suggests defective renal concentrating ability; if azotemic, then renal insufficiency or failure until proven otherwise
	< 1.007	Strongly indicates defective renal concentrating ability but not due to renal failure, as kidneys have ability to dilute ultrafiltrate; consider central or renal diabetes insipidus
Oliguria	> 1.030	Reflects renal attempts to conserve H_2O appropriately
	1.014–1.030	Uncommon; suspect acute renal failure; possibly hypoadrenocorticism
	1.007–1.013	Typical for oliguric renal failure; acute or chronic
Glucosuria	> 1.020[b]	Reflects renal concentrating ability but may be partially impaired by solute diuresis or ↓ medullary hypertonicity (medullary washout)
	1.007–1.020[b]	May reflect impaired concentrating ability caused by solute diuresis or medullary washout but could have concurrent renal insufficiency/failure
Hyponatremia and hypochloremia	> 1.013	Reflects renal concentrating ability but may be impaired by LoH failure as may occur with hypoadrenocorticism
	1.007–1.013	May reflect greater impairment of concentrating ability because of LoH failure but one also must consider renal insufficiency/failure
	< 1.007	Reflects renal diluting ability so not renal insufficiency/failure; ↓ ADH secretion due to hypoosmolality or ↓ medullary tonicity due to LoH failure

[a] If a patient's USG_{ref} is near the guideline limit, then disorders in the adjacent group should also be considered. USG_{ref} values are estimates of urine solute concentrations.

[b] Assuming that the USG_{ref} values are not elevated by either protein or glucose; urine glucose and protein increase the measured USG_{ref} by 0.004–0.005 for every 1 g/dL

Note: Guidelines for cats would reflect the greater concentrating ability of feline kidneys; guidelines for horses and cattle would reflect lesser concentrating ability when challenged or in health.

(3) If there is not azotemia, the finding could represent earlier stages of renal insufficiency or extrarenal disorders that impair renal concentrating ability.

f. USG_{ref} = 1.001–1.015 in a nonazotemic, polyuric animal

(1) Hypothalamic or pituitary disease causing central diabetes insipidus: The disorder decreases the production of ADH, and thus collecting ducts cannot resorb H_2O. There is also reduced absorption of urea in the collecting ducts (less activity of urea-transporter A1 protein).

(2) Hyperadrenocorticism: The pathogenesis of impaired concentrating ability is not firmly established and thus could be either a form of central or nephrogenic diabetes insipidus.

(a) Glucocorticoid hormones inhibited ADH secretion in some studies.[123,124] With lower ADH activity, there is less stimulation of collecting ducts to resorb H_2O.

(b) Cortisol may inhibit the responsiveness of renal tubules to ADH, and thus less H_2O is resorbed.[125] However, some studies indicate that cortisol does not interfere with renal activity of ADH.[123,126]

(3) Hypercalcemia causing nephrogenic diabetes insipidus

(a) Increased [fCa^{2+}] inhibits ADH activity via dysregulation of AQPs. AQP-2 translocation to apical membranes appears to be decreased, and cellular AQP-2 may be degraded by a calcium-sensitive protease.[127–129]

(b) There is also evidence that Ca^{2+} reduces resorption of Na^+ and Cl^- in the ascending limb of the LoH, which reduces the osmotic gradient needed for H_2O resorption in the collecting ducts.[128]

(c) A persistently increased [fCa^{2+}] may cause mineralization of tubular basement membranes, which results in a calcium nephropathy and thus, potentially, renal insufficiency or failure.

(4) Hypoadrenocorticism (Addison's disease)

(a) The USG_{ref} is within this interval in only a small minority of hypoadrenocorticism patients.

(b) The pathogenesis is not well documented, but there may be failure of Na^+ and Cl^- delivery to the LoH and thus failure to maintain medullary hypertonicity, as discussed in the following section, g.

(c) Decreased effective plasma osmolality (because of hyponatremia and hypochloremia) reduces the osmotic stimulus for ADH synthesis and release. Thus, ADH activity might be reduced (conversely, hypovolemia probably stimulates ADH release).

(5) Canine pyometra: The specific pathogenesis is not clear, but the kidneys are refractory or poorly responsive to ADH. One potential mechanism is that bacterial endotoxins initiate the refractory state. Rats made endotoxemic by administration of lipopolysaccharide had decreased urine osmolality with decreased renal ADH receptor and AQP2 expression that might have been mediated by renal inflammatory cytokines.[130]

(6) Hepatic failure: Multiple mechanisms may contribute to defective concentrating ability.

(a) Decreased urea synthesis can lead to a decreased medullary [urea] and thus a decreased medullary concentration gradient.

(b) Other potential contributors include psychogenic polydipsia, defects in portal vein osmoreceptors, and impairment of renal concentrating mechanisms because of increased NH_4^+ excretion.

(7) Hypokalemia due to hyperaldosteronism[131,132] or other disorders that create marked hypokalemia
  (a) Hypokalemia makes collecting ducts less responsive to ADH, in part because of decreased AQP2 expression associated with intracellular AQP2 degradation.[133]
  (b) Low $[K^+]$ in the tubular fluid decreases activities of the Na^+-K^+-$2Cl^-$ cotransporter in the thick ascending limb of the LoH, and of a Na^+/K^+ pump in the collecting duct. With time, the defect results in less Na^+ and Cl^- in the interstitial fluid and thus reduced medullary tonicity.[128,133]

(8) Feline hyperthyroidism: Experimental studies in rats indicate there are two factors that contribute to polyuria. First, hypertension causes more solute to enter tubules and thus contribute to solute diuresis. Second, there is a down-regulation of the AQP-2 channels and thus a poor response to ADH.[134]

(9) Hypoparathyroidism: Polyuria and polydipsia occurred in dogs with primary hypoparathyroidism.[135,136] The pathogenesis of the polyuric state is not understood; it resolved with correction of hypocalcemia.[135]

(10) Psychogenic polydipsia: Excessive H_2O consumption leads to expanded extracellular fluid volume and hypoosmolality. Polyuria results from increased GFR and decreased ADH secretion.

(11) Others: administration of diuretics, alcohol, intravenous fluids, dextrose, or mannitol

g. USG_{ref} < 1.007 to > 1.013 with hyponatremia and hypochloremia
  (1) Because Na^+ and Cl^- are major contributors to the hypertonic interstitial fluid in renal medullae, any disorder that diminishes medullary resorption of Na^+ and Cl^- may diminish renal concentrating ability. The impairment of concentrating ability varies with the degree of reduced medullary hypertonicity. Na^+ and Cl^- resorption in the ascending limb of the LoH may be diminished through two major processes:
    (a) A defective transport system (e.g., inhibition of the Na^+-K^+-$2Cl^-$ cotransporter or Na^+-K^+-ATPase)
    (b) Decreased delivery of Na^+ or Cl^- to the LoH (e.g., persistent hyponatremia or hypochloremia)
  (2) Hyponatremia and hypochloremia also reduce plasma osmolality and thus reduce the stimulus for ADH secretion. Reduced ADH activity in the collecting ducts results in formation of less-concentrated or more-dilute urine.

CHEMICAL EXAMINATION OF URINE (QUALITATIVE OR SEMIQUANTITATIVE)

I. Major concepts
  A. Semiquantitative results of UA procedures (chemical and microscopic) are used to detect or characterize pathologic renal and extrarenal states or to monitor response to therapy. The concentration of a solute in urine depends on two major factors: (i) the amount of solute excreted in the urine over time, and (ii) the amount of H_2O excreted by the urinary system during the same time interval. Solutes from the reproductive tract may alter urine solute concentrations, particularly in voided samples.

1. The semiquantitative results of reagent pad systems are graded on scales provided by the manufacturers of the reagent strips (Table 8.12).
   a. A 1+ result indicates enough solute was present to give a 1+ reaction but not enough to give a 2+ reaction.
   b. When reactions are read by visual examination, distinguishing between a 1+ reaction and a 2+ reaction, or between 2+ and 3+ (etc.), may be difficult. Thus, the true concentration of a solute may be considerably different from the reported value. Inaccuracies may also be caused by the test strips themselves or by automated strip readers.
   c. It is important to store reagent pad strips per manufacturer's recommendations because atmospheric moisture, light, and temperature affect the stability of the reagents in the pads. Routine use of commercial urine control solutions is recommended to help assure properly functioning reagent pads.
   d. Whether urine is applied to each reagent pad by pipette or by briefly immersing the strip in urine, excess urine should be immediately removed by tapping the strip edge against an absorbent material, and the strip should be kept flat; these steps limit

Table 8.12. Semiquantitative values or terms of solute concentrations estimated by UA reagent-strip reactions

	Glucose (mg/dL)	Bilirubin (mg/dL)	Ketone (mg/dL)	Heme[a] (mg/dL)	Protein (mg/dL)	Urobilinogen (mg/dL)
Multistix by Siemens						
Neg.[b]	< 75 to 125	< 0.4 to 0.8	< 5 to 10	< 0.015 to 0.062	15–30	—
Trace	100	—	5	—[c]	10	0.2–1.0
1+	250	Small	15	Small	30	2
2+	500	Moderate	40	Moderate[c]	100	4
3+	1000	Large	80	Large	300	8
4+	≥ 2000	—	160	—	≥ 2000	—
Chemstrip by Roche						
Neg.[d]	< 40	< 0.5	< 9	< 10	< 6	—
Trace	50	—	—	—	6	—
1+	100	0.5	Small	10	30	1
2+	250–500	1.0	Moderate	50	100	4
3+	1000	2.0	Large	250	500	8
4+	—	—	—	—	—	12

[a] The heme test is commonly referred to as either the "blood" or "occult blood" test (see the text for explanation).

[b] Ranges are the reported analytical detection limits of the reactions in contrived urine. Reactions may vary in actual samples. The ketone detection limit was measured with acetoacetic acid. The heme detection limit was measured with hemoglobin (mg/dL). The protein detection limit was measured with albumin.

[c] Reaction pad color changes may be speckled (whole RBCs) or diffuse (free hemoglobin present); reaction charts provided with the reagent strips show trace and moderate speckled (nonhemolyzed) reactions in addition to diffuse reactions.

[d] These values were listed as the detection limits in the package insert.

Note: See the text for pH values.

carryover from one reagent pad to another. If using a pipette, care should be taken to apply sufficient fluid to each pad and to maintain appropriate timing for reading reactions.

2. Reagent pad reactions are designed to be read at specified times after the addition of urine to the pads; reading sooner or later can lead to erroneous results. The color changes can be compared to a color chart provided by the manufacturer (usually on the bottle) or assessed with a reflectance photometer. Automated reflectance photometers are available for photometric assessment of color changes (e.g., the Clinitek family of Urine Chemistry Analyzers). These increase precision of the timing of analysis for each pad and remove the person-to-person variation in the interpretation of color changes on pads. They do not differentiate diffuse and speckled patterns for the heme reaction.
   a. In a study of urine reagent-strip analysis, final-year veterinary students, veterinarians, or veterinary nurses generated more-reproducible results with than without an automated analyzer (Clinitek Status).[137] The study also showed that training is required for appropriate assessment of reagent strips, even when individuals indicate confidence in the procedure.
   b. In a study involving canine urine samples, the authors reported good agreement between visual estimation and automatic measurements (Clinitek 200) for most analytes. The reagent pad values for USG were unreliable.[138]
   c. The cobas u 411 urine analyzer, a semi-automated reflectance photometer developed for human samples and CHEMSTRIP 10UA strips, has been adapted for veterinary use, but it is not designed for species-specific settings.[139] After appropriate instrument adjustments (not possible for pH), concordance of visual and analyzer readings for six analytes in samples from 183 dogs and 95 cats was 62–97 % (mean = 76 %) and 97–99 % if results differing by one unit (e.g., trace vs 1+, 1+ vs 2+) were considered concordant. Accuracy was determined only for [protein], which was assessed in 48 samples by comparison to a turbidimetric benzethonium chloride method; the analyzer results appeared mildly more accurate than the visual results.
   d. Smartphone-based applications to assess color changes have been developed, largely to aid at-home urine assessment.
3. Relationship of urine solute concentration, urine volume, and daily urinary excretion of solutes (Eq. 8.4)

$$\begin{aligned}&\text{Daily urinary solute excretion} = [\text{solute}]_u \times \text{urine volume/d}\\&\text{Examples using protein excretion:}\\&\text{Daily urinary protein excretion} = 70\ \text{mg/dL} \times 1000\ \text{mL/d} = 700\ \text{mg/d}\\&\text{Daily urinary protein excretion} = 140\ \text{mg/dL} \times 500\ \text{mL/d} = 700\ \text{mg/d}\end{aligned} \quad (\mathbf{8.4})$$

   a. If the urine solute concentration remained constant (e.g., 150 mg/dL) from one day to the next but the urine volume doubled (e.g., from 100 mL/d to 200 mL/d), then the urinary excretion of the solute doubled from one day to the next (from 150 mg/d to 300 mg/d).
   b. If the urine solute concentration doubled (from 150 mg/dL to 300 mg/dL) from one day to the next but the urine volume remained constant (100 mL/d), then the urinary excretion of the solute doubled from one day to the next (from 150 mg/d to 300 mg/d).

c. If the urine solute concentration doubled (from 150 mg/dL to 300 mg/dL) from one day to the next but the urine volume halved (from 100 mL/d to 50 mL/d), then the urinary excretion of the solute remained constant from one day to the next (150 mg/d).

4. Relationship of urine volume and USG_{ref} values
   a. If the animal is not in renal insufficiency or failure, urine volume is inversely proportional to USG_{ref}.
   b. If the urinary excretion of solutes remains constant (e.g., 1 g/d) but the urine volume doubles from one day to the next, the USG_{ref} is expected to "halve" from one day to the next (e.g., from 1.040 to 1.020).
   c. If the urinary excretion of solutes remains constant (e.g., 1 g/d) but the urine volume halves from one day to the next, the USG_{ref} is expected to "double" from one day to the next (e.g., from 1.020 to 1.040).
5. Use of concepts to interpret UA results
   a. Dog 1 with a urine [glucose] of 500 mg/dL and a USG_{ref} of 1.015 is typically excreting just as much glucose per day as dog 2 that has a urine [glucose] of 1.0 g/dL and a USG_{ref} of 1.030.
   b. Dog 3 with a urine [protein] of 50 mg/dL and a USG_{ref} of 1.040 is probably not hyperproteinuric. Healthy dogs may have urine protein concentrations of 4–65 mg/dL;[140] USG_{ref} is typically 1.015–1.050. However, dog 4 with a urine [protein] of 50 mg/dL and a USG_{ref} of 1.010 is significantly proteinuric.
   c. Cat 1 with a urine [protein] of 50 mg/dL and a USG_{ref} of 1.010 is typically excreting just as much protein per day as cat 2 that has a urine [protein] of 200 mg/dL and a USG_{ref} of 1.040. Both cats are proteinuric.
6. Conclusions
   a. In results from routine urinalyses, the USG_{ref} helps determine the significance of estimated solute concentrations.
   b. A positive chemical reaction should first be considered a qualitative result (substance likely present); then the significance of the positive reaction can be considered based on reaction strength and the USG_{ref}.

B. The physical and chemical properties of urine may be different after centrifugation if enough particles were suspended in the urine prior to centrifugation.
   1. The following values may be the same before and after centrifugation (unless the heme pigment in RBCs interferes with the reading of color changes):
      a. Color (if due to pigmented solute, not suspended cells or other particles)
      b. USG_{ref} (but the line may be more difficult to read if the urine is cloudy)
      c. pH
      d. Protein
      e. Glucose
      f. Ketone
      g. Heme (if a positive reaction is due to free hemoglobin, free methemoglobin, or myoglobin rather than RBCs)
      h. Bilirubin
   2. The following values may be different before and after centrifugation:
      a. Color, if due to pigmented cells or other suspended particles
      b. Heme, when hemoglobin from RBCs lysed on the reagent pad reacts with the pad
      c. Protein, if RBCs lyse on the pad and release hemoglobin that reacts with the pad

II. pH (negative logarithm of free [H^+]) of urine
  A. Physiologic processes
    1. The pH of urine is affected by many renal and extrarenal factors. Carnivores commonly have acidic urine, whereas herbivores usually have alkaline urine unless they are on milk diets.
    2. Typical urine pH in healthy mammals varies among species: 5.5–7.5 for dogs and cats, 7.5–8.5 for horses and cattle.
    3. Much of the H^+ that is excreted by kidneys is incorporated into other molecules (e.g., NH_4^+, $H_2PO_4^-$, and H_2O).
  B. Reagent-strip method
    1. Principle: A double indicator system is sensitive to change in [H^+]. The indicator does not detect H^+ being excreted in NH_4^+ or $H_2PO_4^-$.
    2. The reagent strip has a pH indicator pad with a range of 5.0–8.5. Results are reported to the nearest 0.5 units (except 5.5 reaction with some strips). The system is designed to provide an estimate of urine pH and does not replace the more precise pH meter when quantitative analysis is desired.[141]
    3. Abnormal urine color (pigmenturia) may interfere with visual interpretation of the color change in the reagent pad.
    4. Contamination with buffer from an adjacent protein reagent pad may falsely decrease the urine pH result.
  C. pH meter method
    1. Principle: The pH is determined based on the difference in electrical potential (voltage) between test and reference electrodes immersed in the urine sample (see Chapter 10); standard laboratory or portable handheld pH meters may be used.[141]
    2. The potentiometric method is the gold standard for measuring [H^+], and it is more accurate and precise than the reagent-strip method; values are reportable to the tenths.[140,141] Because of this, it has been promoted for use when monitoring urine pH during medical management of urolithiasis patients; failure to reach and maintain urine pH targets may promote urolith formation or impair urolith dissolution.
    3. Disadvantages of using a pH meter: additional costs and time for procedure and need for operator training, regular calibration, attention to important maintenance procedures, and, if a portable unit is used, identifying a reliable instrument.
  D. Aciduria may reflect increased secretion of H^+ (Table 8.13)
    1. It is expected in carnivores, some omnivores, and herbivores on a milk diet. Protein-rich diets promote H^+ generation.
    2. Acidemia due to respiratory and most nonrenal metabolic acidoses: There is a net increase in H^+ secretion (H^+ and NH_4^+) by PCTs and collecting ducts as a renal response to acidemia; the secretion is enhanced by decreased extracellular pH (Figure 9.7). Although this response primarily applies to nonrenal acidoses, it occurs in the remaining functional nephrons of animals with renal failure.
    3. Proximal renal tubular acidosis if HCO_3^- depleted: Failure of PCTs to adequately conserve HCO_3^- provides more HCO_3^- to buffer more H^+ in tubular fluid, and thus the urine pH is greater than expected in an acidotic animal (i.e., may be alkalinuric). However, when plasma [HCO_3^-] decreases because of the proximal tubular defect, the remaining tubular function may be enough to conserve the filtered HCO_3^-. Then, there may be insufficient HCO_3^- in the tubular fluid to buffer the H^+, and aciduria may be present.

Table 8.13. Major disorders and conditions which cause abnormal chemistry results in a routine UA
Aciduria
Expected in healthy carnivores and omnivores; herbivores on a milk diet*
Acidemia due to respiratory and most nonrenal metabolic acidoses*
Proximal renal tubular acidosis if HCO_3^- is depleted
Alkalemia associated with hypochloremic metabolic alkalosis ("paradoxical aciduria")*
Hypokalemia
Furosemide therapy
Alkalinuria
Expected in healthy herbivores and after meals in monogastric mammals (alkaline tide)*
Urea degradation:* spontaneous in older samples, initiated by urease-containing bacteria
Alkalemia due to respiratory or metabolic alkalosis
Distal renal tubular acidosis (urine pH also may be inappropriately high but not alkaline)
Proximal tubular acidosis (early)
Proteinuria
Overflow: hemoglobinuria, myoglobinuria, light-chain proteinuria, colostral proteinuria
Glomerular:* glomerulonephritis or amyloidosis
Tubular: congenital or acquired PCT diseases
Hemorrhagic proteinuria*
Inflammatory proteinuria*
Seminal fluid contamination (males)
False-positive reaction (see the text)
Glucosuria (glycosuria)
Hyperglycemia*
Renal: congenital or acquired PCT diseases
False-positive reaction (see the text)
Ketonuria
Ketosis*
False-positive reaction (see the text)
Heme positive[a]
Hematuria (pathologic, iatrogenic, estral hemorrhage)*
Hemoglobinuria and methemoglobinuria from overwhelming intravascular hemolysis*
Myoglobinuria from severe muscle damage
False-positive reaction: bacteria, WBCs, semen, bleach, povidone-iodine
Bilirubinuria
Expected in substantially concentrated urine of healthy dogs*
Hemolytic diseases*
Hepatobiliary diseases (cholestasis) *
False-positive reaction (see the text)

* A relatively common disorder, condition, or mechanism

[a] In most reagent systems, the heme test is called either the "blood" test or the "occult blood" test, although the assay detects heme (peroxidase activity) with or without blood (hematuria may produce a speckled pattern).

4. Alkalemia due to hypochloremic metabolic alkalosis (see paradoxical aciduria and pathogenesis in Chapter 9: Bicarbonate Concentration and Total Carbon Dioxide Concentration, Section III.B.1.a)
5. Hypokalemia: H^+ is secreted and type A intercalated cells resorb K^+ through H^+-K^+-ATPase exchange pumps that appear to be most active when K^+ is depleted.
6. Furosemide therapy: Secretion of H^+ may be increased because of hypokalemia or other factors, e.g., secondary hyperaldosteronism or enhanced renal excretion of Na^+ and H^+ because the Na^+-K^+-$2Cl^-$ cotransporter in the LoH is blocked by furosemide.

E. Alkalinuria may or may not reflect decreased excretion of H^+ (Table 8.13)
1. Urea splitting or hydrolysis: Breakdown of urea releases two $–NH_2$ groups that each quickly accepts H^+ ions to form NH_4^+. Removal of free H^+ from urine makes the urine more alkaline.
   a. It may occur with the spontaneous degradation of urea that occurs with delayed completion of urinalyses.
   b. It may be caused by urease-containing bacteria (e.g., *Staphylococcus, Proteus*, *Corynebacterium urealyticum*), either in vivo or in vitro.
2. Alkalemia due to respiratory or metabolic alkalosis: Probably less H^+ is secreted by the cortical collecting ducts because of less stimulation of the H^+-ATPase pump during alkalemia (Figure 9.4). Also, marked increases in $[HCO_3^-]$ occurring with a metabolic alkalosis can result in excess HCO_3^- in tubular fluid, which could buffer H^+.
3. Distal renal tubular acidosis: Decreased H^+ secretion by the collecting ducts can lead to an inappropriately high urine pH (> 6.0) in the face of acidemia. The pH may not be alkaline.
4. Proximal renal tubular acidosis (see the explanation in the preceding Section D.3)

III. Protein in urine

A. Physiologic processes
1. Many small proteins (usually $M_r < 68{,}000$) can pass through the GFB. In most healthy animals, the proteins are resorbed in the renal tubules (mostly proximal), and thus very little to no protein is detected in urine samples.
2. Urine of healthy dogs may contain a measurable [protein] without clear evidence of urinary tract disease.
   a. Most of the protein is albumin.[142–144]
   b. Dogs (n = 145) with concentrated urine (1.020–1.045) and without evidence of urinary tract disease had negative, trace, or 1+ reactions with a dipstick reagent pad; protein concentrations were 4–65 mg/dL with a Coomassie brilliant blue method and 4–95 mg/dL with a trichloroacetic acid method.[140]
3. Cauxin, a carboxylesterase (M_r = 70) involved in the generation of feline urinary pheromones, is secreted by proximal tubular epithelial cells in quantities reportedly as great as ~100 mg/dL in adult intact male cats, but much less is excreted by females and castrated males.[145]
4. *Uromodulin* (*Tamm–Horsfall protein*) is a mucoprotein that is secreted by the thick ascending limb of the LoH and part of the DCT. It appears to inhibit urolith formation and promote trapping and urinary excretion of bacteria, among other functions.[146] It is part of the matrix of most casts.

B. Analytical concepts
1. Reagent-strip method (see Quantitative Urinalysis for urine albumin methods)

a. Principle (the *protein-error-of-indicator* method): The reagent pad contains a colorimetric pH indicator (tetrabromophenol blue) at acidic pH; amino groups of negatively charged proteins bind the dye, thus changing the pad's color.
b. Changes in pad color correspond to estimated [protein] (Table 8.12).
c. Abnormal urine color (pigmenturia) may interfere with reagent pad color and therefore with estimation of [protein].
d. The potential for falsely increased readings with the protein-error-of-indicator method has been noted for highly buffered alkaline urine,[147] for pH > 9.0 when using the Chemstrip,[148] for moderately alkaline urine if highly concentrated,[149] and for urine that contains quaternary ammonium salts or chlorhexidine. However, relatively little error was detected in alkaline urine in two studies,[149,150] and although addition of sodium carbonate to urine can create false-positive results, physiologic increases in urine pH, total CO_2 concentration, or ionic strength appeared unlikely to interfere with the protein reading for human urine.[151]
e. False-positive reactions may occur if urine contains ciprofloxacin, quinine, or chloroquine.[152]
f. Analytical sensitivity and specificity: It is frequently stated or suggested that the reagent-pad method detects only albumin and does not detect immunoglobulin light chains – published data are not supportive.
   (1) The method detects albumin better than globulins; albumin is most negatively charged, it usually is the most abundant urine protein, and it produces a positive reaction at concentrations lower than most other urine proteins. Proteins within epithelial cells and WBCs react very poorly with reagents; hemoglobin may react if released from RBCs.
   (2) Protein concentrations reported to give a trace to 1+ reaction: albumin (14–21 mg/dL), α globulin (20–30 mg/dL), β globulin (40–50 mg/dL), γ globulin (> 1000 mg/dL), immunoglobulin light chains (26–52 mg/dL), and hemoglobin (5–50 mg/dL)[153,154] However, it was also stated that immunoglobulin light chains are not detected at concentrations up to 150 mg/dL.[151]

2. SSA turbidity
   a. Principle: Proteins are denatured by acids and form a precipitate that is seen as increased solution turbidity after equal volumes of urine and 3 % SSA are mixed. Urine that is hazy to cloudy should be centrifuged prior to SSA turbidity testing.
   b. Results may be expressed on a visual turbidity scale (1+ to 4+) or visually compared against standard solutions to interpolate concentrations; a set of standard solutions is no longer commercially available. A trace-to-4+ system is described.[151] There are also spectrophotometric SSA methods that provide more quantitative results. There is a lack of interlaboratory standardization for reporting SSA test results, and SSA results may not agree well with quantitative results.[150]
   c. SSA reacts with albumin better than globulins (reportedly 2–4 times as well) and detects immunoglobulin light chains if concentrations are sufficient.[151]
   d. Falsely increased readings can be caused by X-ray contrast media, tolbutamide, penicillin (massive dose), sulfisoxazole, tolmetin sodium, and turbidity caused by coprecipitation of crystals because of the low pH of SSA.
   e. Falsely decreased readings can be caused by highly buffered alkaline urine that is not acidified enough by SSA to promote protein precipitation.[106,151,155]
   f. Falsely decreased SSA readings may also occur if there are proteins that are soluble in SSA.[156]

C. Proteinuria (Table 8.13 and Figure 8.12)

1. Overflow (overload, prerenal, preglomerular) proteinuria: A pathologic state increases the plasma concentration of a protein that is small enough to pass through a healthy GFB. If the amount of protein passing through exceeds the ability of PCTs to resorb it, the protein is excreted in the urine. Overflow proteinuria does not produce hypoproteinemia. The ability of urine protein assays to detect these proteins varies (see analytical sections). The proteinuria may be mild to marked.
   a. Hemoglobinuria: During overwhelming intravascular hemolysis, hemoglobin dimers ($M_r \cong 34{,}000$) accumulate in plasma, pass through the GFB, and are incompletely resorbed by overwhelmed renal tubules. Pink to red plasma due to hemoglobinemia is expected if hemoglobinuria is present.
   b. Myoglobinuria: During rhabdomyolysis, myoglobin ($M_r \cong 17{,}000$) from muscle fibers enters plasma, passes through the GFB, and is incompletely resorbed by

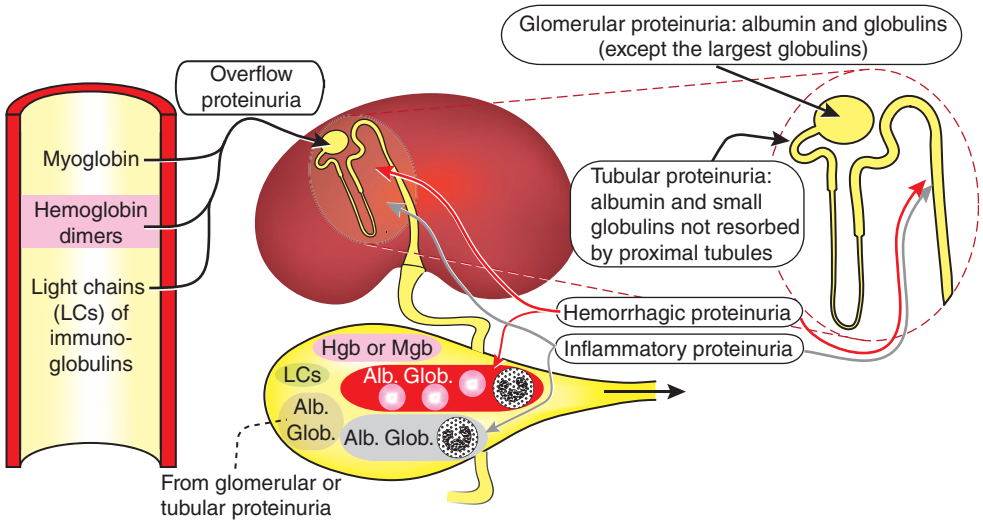


Figure 8.12. Types of proteinuria. Proteins in the urine vary with the pathologic process that is creating the proteinuria. These processes can be organized into the following five types of proteinuria to consider in proteinuric animals.

- Overflow proteinuria: Small proteins are present in plasma at increased concentrations and are excreted in the urine because they pass through the GFB and are incompletely resorbed by tubules. The three causes of pathologic overflow proteinuria are hemoglobinuria, myoglobinuria, and light-chain (Bence Jones) proteinuria.
- Glomerular proteinuria: Glomerular disease damages the GFB and decreases its selective permeability so that it becomes increasingly permeable to larger or negatively charged plasma proteins. These proteins pass through the defective GFB, are incompletely resorbed by tubules, and are excreted in urine.
- Tubular proteinuria: PCTs are defective so that proteins normally resorbed from filtrate (e.g., some albumin and smaller globulins) are not, and thus they are excreted in the urine.
- Hemorrhagic proteinuria: Blood (plasma and cells) enters the urine because of any of a variety of hemostasis defects.
- Inflammatory proteinuria: Plasma proteins enter the urine via inflammatory-mediated exudation.

Hemorrhage or inflammation that creates proteinuria may be in any urinary tract tissue, from the glomerulus out, and potentially in genital tract tissues.

overwhelmed renal tubules. Typically, myoglobin is cleared from plasma so quickly that there is not enough myoglobin present to discolor plasma.

c. Light-chain proteinuria: Immunoglobulin light-chain proteins ($M_r \cong 23{,}000$ for monomers and 46,000 for dimers) are released from neoplastic B lymphocytes or plasma cells, pass through the GFB, and are incompletely resorbed by overwhelmed renal tubules (see Light-chain proteinuria, III.F below).

d. Postcolostral proteinuria in food animals is another type of overflow proteinuria but it does not represent a pathologic state. Proteinuria as great as 3 g/dL is transient, resolving over about 2 d. It consists mostly of low molecular weight proteins that may include intact or altered β-lactoglobulin and α-lactalbumin.[157,158]

2. Glomerular proteinuria
   a. Glomerular disease damages the GFB and decreases selective permeability so that glomeruli become increasingly permeable to larger proteins or to negatively charged proteins. In domestic mammals, the largest globulins (e.g., α_2-macroglobulin) remain in the plasma. Glomerulonephritis and renal amyloidosis are common disorders that create glomerular proteinuria.
   b. Prolonged mild or rapid severe glomerular proteinuria causes selective hypoproteinemia (loss of plasma proteins except for the largest forms) (see Chapter 7) that typically results in hypoalbuminemia without hypoglobulinemia. SDS-PAGE and Bis-Tris-PAGE of urine from dogs with glomerular proteinuria show increased amounts of albumin and larger proteins;[142,159,160] increases in smaller proteins also might be present if tubular resorptive capacity is overwhelmed.
   c. In people, transient proteinuria that occurs after exercise is considered a form of glomerular proteinuria. Exercise-related proteinuria has been reported to occur after dogs swim[161] and after dogs or horses run on a treadmill.[162,163] Clinically significant increases in albuminuria were not detected in dogs that ran 5 km outdoors over 30–40 min at about 25 °C.[164]
3. Tubular proteinuria
   a. PCTs are defective, so proteins that normally are resorbed from filtrate (e.g., some albumin and smaller globulins) are not and thus are excreted in the urine.
   b. SDS-PAGE and Bis-Tris-PAGE of urine from dogs with tubular proteinuria show an increased number and density of protein bands representing proteins with a M_r less than that of albumin.[142,159,160]
   c. Tubular proteinuria is usually associated with acute renal diseases (toxicoses and hypoxia) but can be congenital, including in dogs with mutated genes for the endocytic cubam receptor involved in proximal renal tubular protein resorption and intestinal cobalamin absorption.[165]
   d. Tubular proteinuria does not produce hypoalbuminemia or hypoproteinemia, although these abnormalities may be present for other reasons, including concurrent glomerular proteinuria.
4. Hemorrhagic proteinuria
   a. Hemorrhage into the genitourinary tract can be due to impaired hemostasis, including blood vessel damage by inflammation, trauma, neoplasia, or necrosis. Iatrogenic hematuria is common in urine samples collected via cystocentesis or catheter, but hematuria in such samples may not be iatrogenic. Hematuria (presence of increased RBCs) is expected, although RBCs may lyse and not all be detected on sediment analysis. Heme positivity is expected when assessing whole urine (from hemolyzed or nonhemolyzed cells) and is typically present in supernatant because of RBC hemolysis in vivo or in vitro.

b. The proteins of hemorrhagic proteinuria include the plasma proteins and, if there is RBC lysis, hemoglobin. Proteins from WBCs and platelets are poorly detected by protein assays.
c. It is very unlikely for hemorrhagic proteinuria alone to be severe enough to produce hypoproteinemia.

5. Inflammatory proteinuria
   a. Inflammatory processes involving the genitourinary tract cause exudation of plasma proteins through vessel walls and into the genitourinary tract. Pyuria (presence of increased WBCs) is expected, and it is the laboratory finding that would support inflammation as a cause or contributor to proteinuria. Hematuria may occur concurrently with inflammation.
   b. The quantity of protein lost is usually not sufficient to cause hypoalbuminemia, but there may be mild hypoalbuminemia caused by inflammation. Proteins from WBCs and epithelial cells are poorly detected by urine protein assays.
   c. One must consider reproductive tract sources, especially in voided urine samples.
6. Experimental seminal fluid contamination of canine urine caused trace to moderate increases in urine [protein], with or without spermatozoa.[166] Therefore, urine contamination with seminal fluid and perhaps prostatic fluid may cause or contribute to proteinuria in male dogs.
7. Mixed causes of proteinuria are common, e.g., glomerular and tubular proteinuria or hemorrhagic and inflammatory proteinuria. When proteinuria occurs with hematuria, pyuria, rhabdomyolysis, overwhelming intravascular hemolysis, or a secretory plasma cell neoplasm, proteinuria can be explained and there may be no clinical indication to further assess for renal proteinuria.

D. Other proteinuria classifications: The aforementioned system is not the only system for classifying proteinuria.
1. Some divide proteinuria into three types: (i) *prerenal*, which includes overflow proteinuria, (ii) *renal*, which includes glomerular and tubular proteinuria, and (iii) *postrenal*, which includes hemorrhagic and inflammatory proteinuria. A disadvantage of this classification is that hemorrhagic and inflammatory proteinuria can originate in the kidneys.
2. Another scheme has subdivisions of the renal proteinuria category, i.e., glomerular, tubular, and interstitial subgroups.[167]
3. Hemorrhagic proteinuria and inflammatory proteinuria are sometimes combined and called secretory proteinuria. Renal tubules do secrete uromodulin (Tamm–Horsfall protein) and urinary mucosa can secrete a mucoprotein (especially in horses). However, these secreted proteins are not part of a hemorrhagic or inflammatory proteinuria.
4. *Functional proteinuria* is a renal proteinuria and is considered to be a mild and transient proteinuria caused by physiologically altered renal handling of plasma proteins in the absence of renal lesions (e.g., seen with exercise or fever).

E. Protein-losing nephropathy and renal failure concepts (Figure 8.13)
1. Urea and Crt are small molecules that pass freely through the GFB (sieves). Some filtrate urea is resorbed by tubules. The remaining urea and Crt are excreted in urine and thus do not accumulate in blood. Very little albumin passes through the GFB in health, and almost all that does is resorbed by tubular epithelium, so essentially all plasma albumin remains in blood.
2. With a protein-losing nephropathy, the glomerular "sieves" become more porous, and larger proteins or charged proteins that usually are repelled enter the renal filtrate via

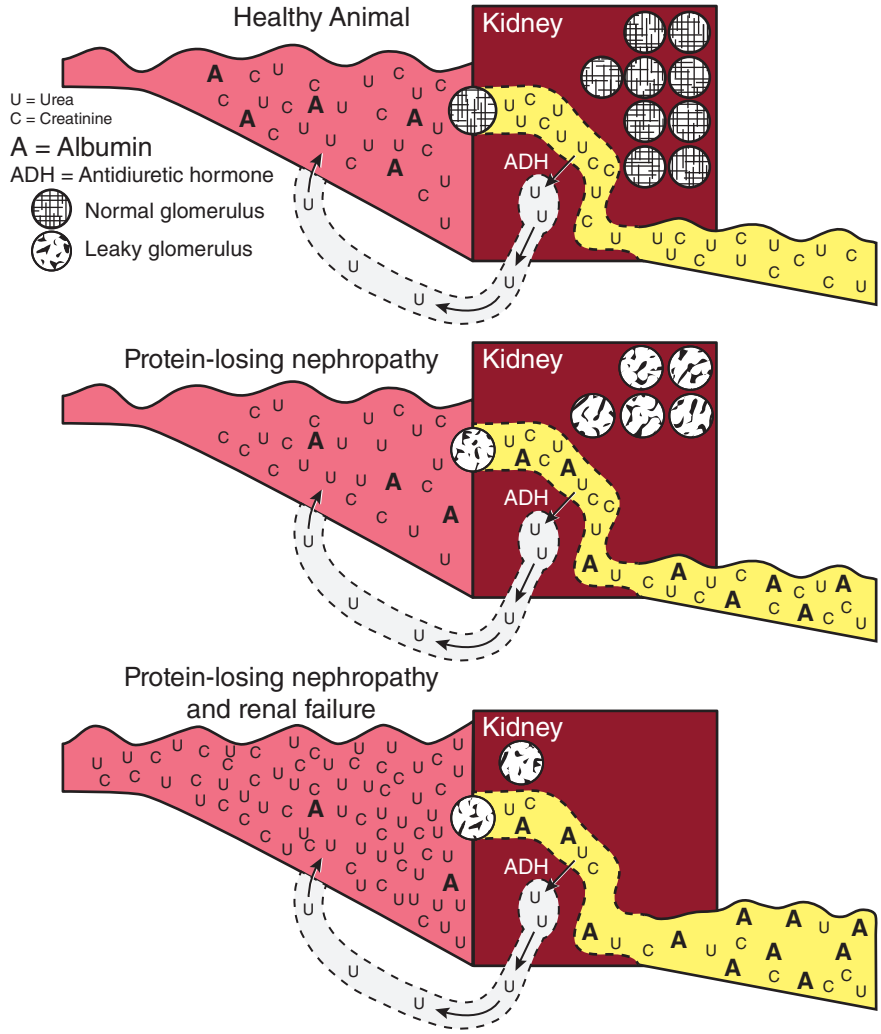


Figure 8.13. Protein-losing nephropathy and renal failure. Illustrations depict a body's extracellular fluid as a lake, kidneys as a dam, and the urine as the river below the dam. Urea and Crt molecules are small fish in the lake, and albumin molecules are big fish. The nephron consists of the sieve or filter at the lake outlet and the tubes that run through the dam. Some H_2O and urea are reclaimed to maintain extracellular fluid H_2O and urea content. Only 1 nephron per dam is shown as a filter with a connected tubular system, but each would have one.

- In healthy animals, urea and Crt pass freely through 10 functional filters, into the dam's tubes, and into the river. Some urea (and H_2O) is reclaimed from the dam's tubes in a process enhanced by ADH. Albumin is too big to pass through the filters, so it stays in the lake.
- In the protein-losing nephropathy illustration, 40 % of the filters (and nephrons) have been destroyed. The remaining 60 % are damaged, more porous, and allow albumin to enter the dam and river. The loss of albumin from the lake causes hypoalbuminemia and proteinuria. The remaining filters are sufficient to keep urea and Crt removed from the lake, and thus azotemia does not develop.
- In protein-losing nephropathy and renal failure, 80 % of the filters (and nephrons) have been destroyed. The remaining 20 % are damaged, more porous, and allow albumin to enter the dam and river. The loss of albumin from the lake causes hypoalbuminemia and proteinuria. The remaining filters are insufficient to keep urea and Crt removed from the lake, and thus azotemia develops. Also, the remaining nephrons cannot adequately conserve H_2O, and thus polyuria develops. The "lake" is higher to represent the expanded extracellular fluid volume that occurs with retention of Na^+ and H_2O that can lead to edema and a protein-poor transudate in the peritoneal cavity.

glomeruli. If the ability to resorb proteins is exceeded, then proteinuria occurs. The continual loss of protein may lead to hypoalbuminemia. As long as the number of functional glomeruli is adequate, urea and Crt are adequately removed from blood and azotemia does not develop.

3. If the glomerular disease destroys more nephrons, then renal failure occurs. The few remaining functional glomeruli cannot remove urea and Crt fast enough from the blood, so azotemia develops. Proteinuria continues because the remaining functional glomeruli are permeable to proteins. The severity of hypoalbuminemia increases because of continued albumin loss, but defective excretion of H_2O (associated with Na^+ and H_2O retention) may contribute to hypoalbuminemia.

F. Light-chain proteinuria (Bence Jones proteinuria)

1. Analysis of urine for immunoglobulin light chains is not part of a routine UA. It may be indicated to clarify the type of proteinuria or to investigate a possible lymphoproliferative disease. Free light-chain proteinuria is the most common form of paraproteinuria, but paraproteinuria has been reported with immunoglobulin heavy chains and no free light chains.[168]
2. Light-chain proteinuria occurs when the formation of κ- or λ-light-chain proteins by lymphocytes or plasma cells is increased enough.
   a. Typically, their presence is associated with B lymphocyte or plasma cell neoplastic disorders.
   b. Light-chain proteinuria can also be caused by B-lymphocyte hyperplasia. Based on SDS-PAGE and immunofixation methods, light-chain proteinuria was reported in dogs with leishmaniasis, ehrlichiosis, and babesiosis.[169]
3. *Bence Jones proteins*[1] are free light chains (either κ or λ) of immunoglobulins that have unique thermal properties: they precipitate between 40 °C and 60 °C, return to a soluble state at 100 °C, and then precipitate again when cooled. The thermal properties of Bence Jones proteins are due to the variable portions of the light-chain proteins.[171]
4. The unique thermal properties of light-chain proteins are used as the basis of the Bence Jones test. It is appropriate to describe the proteinuria as a Bence Jones proteinuria when the thermal test is positive.
   a. Basics of the positive heat test:[108] (i) Acidified urine (pH 4.8–5.0) is heated to 56 °C for 15 min and observed for flocculence or precipitates. (ii) If flocculence or precipitates are present (the test is negative if they are absent), the urine sample is placed in a boiling water bath for 3 min and examined for a decreased amount of precipitate. A decrease in flocculence is a positive result that suggests Bence Jones proteinuria. (iii) If there is not a decrease in flocculence, the hot urine is filtered through a funnel with filter paper into a tube containing a thermometer. This is done to remove other precipitated proteins that may be masking the presence of Bence Jones proteins. If Bence Jones proteins are present, they should precipitate at about 60 °C and redissolve at about 40 °C. Variations in the heat test acidifying solutions, filtrations, and time intervals have been described.

[1] As described by Ritzman and Daniels,[170] these proteins were first recognized by William MacIntyre in 1845. Dr. MacIntyre sent the urine sample to Dr. Henry Bence Jones (not Bence-Jones), who confirmed the findings and examined the urinary protein in detail. Because Dr. Jones reported on the properties, analysis, and diagnostic significance of the finding in 1847, and McIntyre focused on clinical aspects of myeloma in his publication in 1850, these proteins came to be known as "Bence Jones proteins" and not "MacIntyre proteins," this term being first used in 1880, seven years after Dr. Bence Jones had died.

b. The concentration of light-chain proteins needs to be > 145 mg/dL for a positive Bence Jones test result, and regulation of the pH is very important.[171]
c. Because other proteins may precipitate during heating (e.g., fibrinogen precipitates at 56–58 °C) and not dissolve at 100 °C, the filtering step is necessary, and this requires a sufficient volume of urine (5 mL or more).

5. Other methods of detecting light-chain proteinuria
a. Immunoglobulin light chains react better in the SSA protein method than they do in the protein reagent pad method of routine UA procedures.[171] The reagent pad test may fail to detect light-chain proteinuria if the proteinuria is minimal.
b. If urine proteins are concentrated prior to electrophoresis, light-chain proteins can be found anywhere from the α_1 region to the γ region.[172] The light-chain proteins may be represented by a single band or may be migrating with other urine proteins and thus not recognized by routine urine protein electrophoresis.
c. Immunoelectrophoresis for κ or λ free light-chain proteins is the preferred method of documenting light-chain proteinuria, but species-specific antisera are not available, and antibodies for human free light chains have limitations (see Chapter 7).[173]
d. Quantitative assays for urine proteins differ in their abilities to react with immunoglobulin light chains. The dye-bindings assays (Coomassie brilliant blue, pyrocatechol red molybdate) detect the light chains better than the precipitant assays (see Quantitative Urinalysis).[174–176]

IV. Glucose in urine
A. Physiologic processes
1. Glucose is a relatively small molecule (M_r = 180) that passes freely through the GFB and enters the filtrate.
2. Glucose is resorbed in the PCTs via a Na^+-glucose cotransport system that depends on a favorable gradient established by active Na^+ transport from tubular cells to the peritubular fluid. The gradient promotes Na^+ resorption from the tubular fluid, and glucose is resorbed secondarily (indirect or secondary active transport).
3. The renal tubular transport maximum for glucose varies among domestic mammals. The approximate transport maximums are 180–220 mg/dL in dogs,[177] ~290 mg/dL in cats,[177] ~150 mg/dL in horses[178] and calves,[179] and probably lower in mature cattle. Data were not found for alpacas and llamas, but glucosuria (2+ to 4+) is reportedly common in llamas with blood [glucose] > 270 mg/dL,[180] and the glucose transport maximum has been suspected to be in the vicinity of 150–180 mg/dL.

B. Analytical concepts
1. Reagent-strip method
a. Principle: Conversion of glucose to gluconic acid is catalyzed by glucose oxidase with liberation of H_2O_2, which reacts with an indicator to give a color change in the reagent pad. The degree of color change is proportional to the [glucose] (Table 8.12). Indicators vary with different products.
b. Falsely increased reactions may be caused by H_2O_2 and sodium hypochlorite (bleach). Such contamination may occur when samples are obtained from an examination table or cage floor.
c. Falsely decreased reactions may be caused by ascorbic acid, ketones, and very concentrated urine samples, particularly when alkaline. Cold urine and marked bilirubinuria may also inhibit reactions. If samples were refrigerated, they should be warmed to room temperature before testing.

2. Copper-reduction method (Clinitest)
   a. Principle: Cu^{2+} reacts with a reducing substance (e.g., glucose, fructose, lactose, galactose, maltose, or pentose) to produce cuprous (Cu^{+}) oxide and cuprous hydroxide and thus a color change.
   b. Semiquantitative results for the standard method are negative, ~250, ~500, ~750, ~1000, or ~2000 mg/dL. The method is probably more accurate than the reagent-strip method but requires a greater concentration for detection, i.e., the detection limit is higher.
   c. The method may be used to confirm questionable positive reagent-strip results such as when urine color interferes with interpretation of the reagent pad color.
   d. False-positive reactions may be caused by cephalosporins, formaldehyde, and ascorbic acid (if concentrations are high enough). Reactions with sugars other than glucose might be considered false-positive reactions.

C. Glucosuria (glycosuria) disorders (Table 8.13)
1. The term *glucosuria* is generally applied when glucose is detected by reagent strips or pads used for routine UA. However, glucose concentrations below limits of detection for these methods (Table 8.12) are usually present in at least dogs, cats, and people.[181,182] These low concentrations (usually < 20 mg/dL) in urine from healthy individuals have been called *basal glucosuria*. In few cats and dogs, urine reagent strips with [glucose] detection limits of 20 mg/dL might yield slight positive results in the absence of disease.
2. Hyperglycemic glucosuria
   a. Transient or persistent hyperglycemia results in more glucose in the filtrate than can be resorbed by PCTs.
   b. Hyperglycemia is typically concurrent with the glucosuria, but a transient hyperglycemia and delay in bladder emptying may mask concurrence.
   c. Stress-induced hyperglycemic glucosuria is common in camelids.[14]
3. Renal glucosuria (also called normoglycemic glucosuria, though it may occur with hypoglycemia too)
   a. Transient or persistent glucosuria results from defective resorption of glucose caused by damaged or abnormal PCTs.
   b. Tubular abnormalities can be acquired or congenital
      (1) Acquired: Acquired proximal renal tubular dysfunction (sometimes referred to as *acquired Fanconi syndrome*) can occur with ischemia and certain toxicants or infections. It occurs in some dogs with copper-associated hepatitis,[183] and has been associated with consumption of dried chicken treats (jerky)[184–186] and leptospirosis.[187] Administration of a pharmacologic inhibitor of Na^{+}-glucose cotransporter 2 (developed to manage diabetic nephropathy in people) would also specifically promote glucosuria.
      (2) Congenital: Fanconi syndrome and pure primary renal glucosuria (basenji, Norwegian elkhound, and Shetland sheepdog)[188]
4. Glucose in tubular fluid causes osmotic diuresis (H_2O is "held" by the glucose in the tubular fluid, especially in the PCTs) and thus may cause decreased renal concentrating ability and increased urine volume (polyuria).

V. Ketones in urine
A. Physiologic processes

1. Acetoacetate, β-hydroxybutyrate, and acetone are ketone bodies, but only acetoacetate and acetone have the chemical structure of ketones (see Chapter 9). Acetoacetic acid and β-hydroxybutyric acid are ketoacids, but they dissociate at physiologic pH to their anionic form (conjugate base) and H^+.
2. Ketone bodies are not expected in the urine of healthy mammals that have an adequate intake of nutrients.
3. Ketone bodies freely enter the urine by glomerular filtration of plasma. After entering the tubular fluid, acetoacetate and β-hydroxybutyrate are resorbed by monocarboxylate transporters until their transport maximums are exceeded.

B. Analytical concepts
   1. Reagent-strip method (Siemens)
      a. Principle: Acetoacetate (mostly) and acetone (about 10 % as reactive as acetoacetate) form colored complexes with nitroprusside. The amount of color change reflects the amount of ketones present (Table 8.12). The reagent system does not react with β-hydroxybutyrate, the ketone body that is not a ketone (does not have a ketone chemical structure).
      b. False-positive reactions may be caused by highly pigmented urine, levodopa metabolites, and some compounds that have sulfhydryl groups (e.g., captopril, sodium 2-mercaptoethanesulfonate, and N-acetylcysteine). Trace reactions may occur in urine with high USG and low pH.
      c. In theory, β-hydroxybutyrate can be converted with H_2O_2 to acetoacetate so that nitroprusside methods can be used to detect β-hydroxybutyrate. However, urine concentrations of β-hydroxybutyrate must be > 200 mmol/L (using 3 % H_2O_2) or > 50 mmol/L (using 30 % H_2O_2) to produce more than a trace reaction. At such concentrations, the [acetoacetate] would probably be great enough to be detected routinely.[189]
   2. The nitroprusside reaction is not specific for acetoacetate and acetone; other ketones react. Of three compounds that are called phenylketones, 4-hydroxyphenylpyruvate (a true ketone) reacted with nitroprusside to produce a red-brown color (acetoacetate produces a maroon color), but 4-hydroxyphenylacetate and 4-hydroxyphenyllactate do not have a ketone structure and did not react.[190] Some considered the reaction with 4-hydroxyphenylpyruvate a false positive for ketonuria, but the assay did detect ketonuria – just not ketonuria created by acetoacetate or acetone.
   3. Acetest tablet method
      a. Principle: It is the same as with the reagent-strip method. Color change is easier to detect, so it has a lower detection limit than some reagent pads (~5 mg/dL in urine) and thus may be used to confirm trace or questionable reactions on reagent pads.
      b. The method may be used as a qualitative assay for blood, plasma, urine, and milk.

C. Ketonuria (Table 8.13)
   1. *Ketonuria* occurs when the mobilization of lipids is increased because of a shift in energy production from carbohydrates to lipids (e.g., in diabetes mellitus, anorexia, starvation, and hypoglycemic disorders). Excessive β-oxidation of fatty acids in hepatocytes generates more acetyl-coenzyme A than can be used for gluconeogenesis and triglyceride synthesis. Excess acetyl-coenzyme A stimulates hepatic ketogenesis and thus increased formation of ketone bodies leading to increased ketone bodies in blood *(ketonemia)*. Ketone bodies are easily cleared from blood and are excreted in urine.
   2. Decreased insulin activity and increased glucagon activity promote ketogenesis. Such changes may result from either physiologic or pathologic processes.

D. The pathologic state caused by excess ketogenesis is called *ketosis* or, if laboratory data indicate a concurrent metabolic acidosis, *ketoacidosis*. In ketosis, primarily β-hydroxybutyrate (a ketone body, not a ketone, and therefore not detected by the assay) is excreted in urine, but acetoacetate and acetone (ketones detected by the assay) are also excreted.

E. Renal excretion of acetoacetate and β-hydroxybutyrate (both of which are anions) obligates excretion of cations (e.g., Na^+ or K^+) by the kidneys. Prolonged ketonuria may cause Na^+ or K^+ loss that contributes to hyponatremia and hypokalemia.

VI. Heme in urine (test frequently labeled *blood* or *occult blood*)

A. Physiologic concepts: Heme-containing compounds are not expected to be present at detectable amounts in the urine of healthy mammals.

B. Labeling this analyte *blood* is misleading because blood does not need to be present for a positive reaction. Similarly, although the reaction pad is very sensitive and can detect small amounts of blood that are not evident macroscopically by a reddish color (i.e., occult blood), *occult blood* is also a misleading label as it implies blood is present but cannot be seen.

C. Analytical concepts

1. Reagent strip method
   a. Principle: In some systems, the peroxidase activity of heme catalyzes the oxidation of o-toluidine to a blue compound. In other systems, the peroxidase activity of heme catalyzes the oxidation of a chromogen (e.g., tetramethylbenzadine) to a colored compound.
   b. Heme may be from hemoglobin, methemoglobin, or myoglobin. If intact RBCs are present in whole urine, the cells are lysed on the reagent pad to produce either speckled or solid color changes in the pad. Results may be described in semiquantitative terms or as an estimated [hemoglobin] (Table 8.12).
   c. False-positive reactions for heme may occur with oxidizing compounds such as sodium hypochlorite (bleach) and with peroxidase activity from urinary microbes, WBCs, or semen.[166] A positive-reaction also occurs with povidone-iodine (Betadine) contamination.[191]
   d. Falsely decreased reactions may occur with a high USG or with urine that contains captopril or formaldehyde. RBCs may not be detected if they are not suspended in the test sample.
2. Hematest tablet method
   a. Principle: It is the same as for the reagent pad, with modifications.
   b. The method may be used as a confirmatory test and also to test for fecal occult blood.
3. Unfortunately, there is not a widely available laboratory assay for differentiating myoglobin from hemoglobin in domestic mammals. Differentiation is usually accomplished by clinical deductive reasoning (Table 3.13).
   a. A simple precipitant test using ammonium sulfate is considered unreliable. In theory, hemoglobin, but not myoglobin, precipitates in 80 % ammonium sulfate solution, whereas myoglobin precipitates in a 100 % ammonium sulfate solution. However, denatured myoglobin precipitates in the 80 % solution and thus would be incorrectly classified as hemoglobin.[192] Also, a similar erroneous classification occurs if the urine pH is not adjusted to 7.0–7.5.[193]

b. Immunologic methods require species-specific antibodies. Electrophoretic and spectrophotometric procedures may be employed.

4. The heme assay is more sensitive than the protein assay. Therefore, when small amounts of blood are in the urine, there may be marked heme positivity without the hemoglobin causing a positive protein reaction.

D. Heme-positive reaction in urine (Tables 8.13 and 3.13)

1. Hematuria (bleeding, hemorrhage)
   a. RBCs enter the urine via hemorrhage into the urogenital tract. Hemorrhage may be caused by a pathologic process (trauma, inflammation, neoplasia, coagulation defect, etc.) or the urine collection process (e.g., cystocentesis, catheterization, or bladder palpation pressure).
   b. According to package inserts from multiple companies, positive reactions can occur with as few as 5 RBC/μL when RBCs are intact, and with fewer RBCs if RBCs have lysed and thus released hemoglobin to react with the reagent pad. Confirmation of hematuria requires identifying RBCs microscopically in the urine sediment. RBCs tend to lyse if urine is very alkaline or not more than mildly concentrated (e.g., $USG_{ref} < 1.015$); intact RBCs may not be seen on examination of the sediment, but RBC ghosts may be present.
2. Hemoglobinuria (renal excretion of hemoglobin that was in plasma)
   a. During overwhelming intravascular hemolysis, when haptoglobin is saturated, plasma hemoglobin dimers ($M_r \cong 32{,}000$) forming from hemoglobin ($M_r \cong 64{,}000$) pass through the GFB and enter the filtrate (see Chapter 3). If not completely resorbed by the renal tubules, the hemoglobin dimers are excreted in the urine.
   b. Intravascular hemolysis of sufficient severity to cause hemoglobinemia and hemoglobinuria can be caused by a variety of disorders that cause anemia (Tables 3.12 and 3.14).
   c. Hemoglobin that is free in the urine from lysis of RBCs in the urine (i.e., hemorrhage) might also be called hemoglobinuria, but *hemoglobinuria* is typically used for the presence of hemoglobin from intravascular hemolysis, not from hemorrhage, and it may be defined as the excretion of plasma hemoglobin into urine (not just its presence).
   d. Methemoglobin may contribute to a positive urine heme (metheme) reaction[194] in the following situations.
      (1) When hemoglobin (or hemoglobin dimer) molecules are in the urine, some of the ferrous heme spontaneously oxidizes to ferric heme (forming methemoglobin).
      (2) When there is an intravascular hemolytic anemia caused by oxidative injury, some of the lysed RBCs release methemoglobin to plasma. Then, methemoglobin dimers can enter the glomerular filtrate.
3. Myoglobinuria (renal excretion of myoglobin that was in plasma)
   a. Myocyte necrosis or sublethal damage can release myoglobin ($M_r \cong 17{,}000$) from the myocyte into the interstitial fluid, lymph, and finally blood, from which the small protein easily passes into the glomerular filtrate. If not completely resorbed by the renal tubules, myoglobin is excreted in the urine.
   b. Myoglobin is rapidly cleared from plasma, and thus pink plasma is not expected. Myoglobinuria is associated with acute myopathies caused by trauma, excessive exertion, or various other forms of rhabdomyolysis, particularly in horses.

4. Not uncommonly, a positive heme reaction is present without other evidence of hematuria, hemoglobinuria, or myoglobinuria. If pyuria, bacteriuria, or spermatozoa are present, such reactions could be caused by peroxidase activity in WBCs, bacteria, or semen, respectively. Seminal fluid might also contribute to an increased heme reaction in the absence of spermatozoa.[166] Consider sample contamination with bleach or other oxidizing substance, and lysis of RBCs in urine without increased RBCs on sediment exam.

VII. Bilirubin in urine

A. Physiologic processes

1. Bilirubin is not expected in the urine of domestic mammals other than the dog.
2. Bu forms from the degradation of heme, primarily from hemoglobin degradation in macrophages. After conjugation in hepatocytes, Bc is excreted via the biliary system. If biliary excretion is overwhelmed or impaired and Bc is transported to the plasma, Bc passes freely through the GFB and is excreted in urine (Figure 3.3 or 13.1).
3. Renal tubular cells in at least some species (e.g., dogs) can convert heme (from resorbed hemoglobin dimers of hemoglobinuria) to Bu and then to Bc, which can be excreted in the urine.
4. Usually, Bu is thought not to be in urine because it is bound to plasma albumin, which does not pass through most GFBs. However, because apparently healthy dogs may have mild albuminuria and diseases can cause proteinuria, Bu bound to albumin may be present in urine. Also, Bc may hydrolyze to Bu in urine.

B. Analytical concepts

1. Reagent-strip method
   a. Principle: Bilirubin becomes coupled with a diazonium salt in a strongly acid medium, and the coupling produces color changes that reflect the amount of bilirubin present (Table 8.12). The reagent pad lacks the accelerators used to quickly detect Bu in serum or plasma assays, so detection of Bu is not expected. Biliverdin (an intermediate breakdown product between heme and bilirubin) is not detected.
   b. Color changes with this reagent pad are often difficult to interpret as they do not clearly match the reference colors provided with the test strips. The Ictotest may be used as a confirmatory method for suspected positive reactions.
   c. Falsely increased reactions may be caused by indican (a product of tryptophan degradation by intestinal bacteria) and metabolites of etodolac (a nonsteroidal anti-inflammatory drug).
   d. Falsely decreased reactions may be caused by ascorbic acid if the animal is being given ascorbic acid (vitamin C).[108]
2. Ictotest method: Although based on the same analytical principle, it has a lower detection limit (about 0.1 that of reagent strips) and may be used to confirm trace or questionable reactions on reagent sticks. Reagent pad results can be reported when the Ictotest confirms positivity.
3. Light in primarily the blue region of the visible spectrum degrades Bu and Bc to various isomers and oxidative products that assays do not detect, thus decreasing measured concentrations. Direct sunlight has a rapid and marked effect, whereas clinically significant changes in bilirubin concentration are unlikely under routine laboratory conditions with fluorescent lighting and sample analysis within 2 h[195] (and unpublished data, M. Gois Ceregatti and MAS).

C. Bilirubinuria (Table 8.13)
   1. Excessive hepatic bilirubin formation (in hemolytic states) or impaired hepatobiliary excretion of Bc due to cholestasis increases plasma [Bc]. Bc passes easily through the GFB and is excreted by the kidneys.
   2. Intravenous injections of Bu resulted in the excretion of Bc by the isolated canine kidney.[196] Thus, the bilirubinuria that occurs with extravascular hemolysis may reflect the conjugation of bilirubin by the liver, the kidneys, or both. Similarly, hemoglobinemia may lead to bilirubinuria by the renal tubular degradation of heme and conjugation of bilirubin.
   3. In dogs, a positive bilirubin reaction must be interpreted based on knowledge of the USG_{ref}. Concentrated urine (1.025–1.040) of healthy dogs frequently produces a small bilirubin reaction, and moderate reactions occasionally occur with more concentrated urine (> 1.040).
   4. Because of the low renal threshold for bilirubin in most animals, especially dogs, bilirubinuria may be detected before hyperbilirubinemia or icterus is detected in hemolytic and hepatobiliary disorders. Hyperbilirubinemia without bilirubinuria suggests a false-negative urine bilirubin reaction or the degradation of urine bilirubin before sample analysis.
   5. In the authors' experience, bilirubin reactions are sometimes negative when bilirubin crystals, and therefore bilirubinuria, are present. The reason is unknown. Potential explanations include too little remaining soluble bilirubin to be detected because it has been incorporated into crystals, or chemical alteration of bilirubin to an unreactive form.

VIII. Urobilinogen in urine
   A. Physiologic processes
      1. *Urobilinogen*, a colorless compound formed from the degradation of bilirubin in the intestine, is passively absorbed by intestinal mucosa.
      2. Urobilinogen that is not removed from portal blood by hepatocytes enters the systemic blood, from which it is excreted in the urine.
   B. Analytical concepts for the reagent-strip method
      1. Principle: Urobilinogen reacts with p-dimethylaminobenzaldehyde to form a red pigment via a modified Ehrlich's aldehyde reaction. The degree of color change reflects the [urobilinogen] (Table 8.12).
      2. Units: Ehrlich units (EU) or mg/dL; 1 EU = 1 mg/dL
      3. The assay cannot be used to detect decreased urobilinogen concentrations because the lowest reading is 0.2 mg/dL (Multistix) or 1.0 mg/dL (Chemstrip), the concentration expected in urine of healthy animals.
      4. Falsely increased results may be caused by aminosalicylic acid, sulfonamides, and aminobenzoic acid.
      5. Falsely decreased reactions may be caused by formalin. In addition, urobilinogen degrades easily when urine is exposed to light.
   C. Increased urobilinogenuria
      1. Hemolytic diseases cause increased formation, biliary excretion, and intestinal degradation of bilirubin. Thus, increased urobilinogen formation and urinary excretion are expected. However, increased urobilinogenuria is not consistently found.
      2. Assessment of urine [urobilinogen] is of little diagnostic value in veterinary medicine.

IX. Nitrite in the urine
- A. Physiologic processes: Certain Gram-negative bacteria can reduce nitrate to nitrite.
- B. Analytical concepts for the reagent-strip method
  1. Principle: Nitrite reacts with p-arsanilic acid (or other amine) to form a diazonium compound that couples with N-1-naphthyl ethylenediamine (or other compound) to form a pink product.
  2. Falsely increased reactions may result from analysis of stale urine containing contaminant bacteria.
  3. Falsely decreased reactions for bacteriuria (but correct results for absence of nitrite) may occur if there is insufficient time (< 4 h) for bacterial reduction of nitrates. Ascorbic acid (especially when USG is high) may produce falsely decreased reactions in dog and cat urine.[106]
- C. Nitrite-positive reaction
  1. Suggestive of a Gram-negative bacterial infection in the urinary tract
  2. Detection of nitrites in urine is a screening procedure for Gram-negative bacterial urinary infections. Significant bacteriuria may be present but undetected with the nitrite assay.
- D. Most veterinarians do not find the urine nitrite test to be diagnostically valuable because it does not detect significant bacteriuria consistently. In a study that included 29 cultured canine urine samples including 15 that were positive for a total of seven bacterial genera (mostly Gram negative), each of four brands of reagent strips detected too few positive results (0–3) to be considered useful.[197]

X. Leukocyte esterase in the urine
- A. Physiologic processes: WBCs in the urine may release esterases.
- B. Analytical concept: Hydrolysis of reagent pad esters by urine esterases forms a compound that reacts with a diazonium salt to yield a purple color.
- C. The assay has been considered inadequate for the chemical detection of pyuria in dogs because of too many false-negative results.[198] In a study of 112–116 canine urine samples, 19 of which were pyuric based on routine UA, use of manufacturers' recommendations for four brands of reagent strips yielded diagnostic sensitivities for pyuria of only 32–53 % (many false-negative results).[197] However, diagnostic specificities were 97–100 %, indicating that pyuria was very likely when a result was positive. By changing the protocol to use of resuspended urine sediment instead of whole urine, reading the reaction after 150 s instead of 120 s, and using a trace reaction rather than a 1+ reaction as the threshold for positive, the diagnostic sensitivity for pyuria increased to 67–90 % while the diagnostic specificity decreased to 80–89 %.
- D. Many false-positive reactions are a problem with feline urine.[108,197]

URINE SEDIMENT EXAMINATION

I. General concepts for traditional approach
- A. The contents of urine are not stable, so urine should be analyzed within a few hours (ideally within 1 h) of collection. During sample storage, cells and casts deteriorate, crystals may dissolve or form, and bacteria may die or proliferate.
- B. The method of urine collection should be recorded so that it can be considered when UA and especially urine sediment results are interpreted. A voided sample may contain cells and

bacteria from the genital tract. A catheterized sample may contain more epithelial cells or RBCs because of urethral trauma. A cystocentesis sample may contain blood because the needle damaged blood vessels.

C. Consistently using the same urine volume for sediment preparation enables a more critical assessment of the semiquantitative results. About twice the amount of sedimented material would be expected from 10 mL than from 5 mL. Expected findings in this chapter are based on the sedimentation of 5 mL of urine, a volume that is more practical than 10 mL but certainly not always achievable.

D. Samples should be well mixed before aliquots are removed for centrifugation in standardized conical-based tubes at ~450 × g (~1600–1700 rpm for centrifuges with a 5.5–6 in radial arm) for 5 min without braking. Higher centrifugal force can pack structures too tight (making resuspension difficult) or damage fragile structures. Compared to this traditional approach, recovery of cells was lower when preparations were made after centrifugation of only 1.5 mL mock urine for 45 s at 3900 × g (StatSpin VT).[199]

E. The supernatant is poured off (decanted) or removed by pipette to leave a residual urine volume (commonly 0.2–0.5 mL) in which the sedimented material should be thoroughly resuspended by pipette or by repeatedly flicking the base of the tube. A standardized drop is then placed on a slide with a pipette and overlaid with a square glass coverslip; the drop should fill the space beneath the coverslip, with no or minimal air bubbles, and not overflow the coverslip borders. Note: With some UA systems, as much as 1 mL of fluid may be left after decanting, and resuspended sediment is quantitatively pipetted into standardized wells with counting grids for microscopic analysis.

F. The sediment should be analyzed after a few minutes (to let cells and other structures settle and lipid float) by using 10× and 40× (or 45×) objectives with the microscope condenser lowered or the iris diaphragm (condenser diaphragm) partially closed to increase refraction. At least 10 fields should be assessed at low and at high magnification, including edges of the preparation where larger formed elements tend to distribute. Most sediment findings are quantified by the average number or range of numbers of structures seen per microscopic field (i.e., per lpf or hpf). The width of the viewed field can vary because of differences in the microscope objective and ocular lens. For example, the viewed area of a 25 mm ocular is about 50 % larger than the viewed area of a 20 mm ocular. Thus, in the same sediment, 15 WBCs/hpf would be seen with a 25 mm ocular, and 10 WBCs/hpf would be seen with a 20 mm ocular.

G. Sediment stains may be used but could affect concentrations via dilution and may introduce organisms or particulate debris. Evaluation of stained and unstained preparations may be helpful.

1. For staining, one drop of a commercial stain (Sedi Stain) or new methylene blue may be added to resuspended sediment and briefly incubated prior to making a wet preparation as described above. Stained and unstained preparations fit side-by-side on one slide.
2. The stain may help identify nucleated cells and other structures, but bacteria do not necessarily stain. The package insert for Sedi Stain (Clay Adams) cites Sternheimer[200] in the performance characteristics section, which states: "dead bacteria stain dark purple, while living bacteria remained either unstained or stained pink."[201]

H. When the nature of formed elements is unclear from wet preparations, air-dried cytocentrifuge or flowback preparations of sediment can be made and stained with a routine Romanowsky stain. Although cell deterioration can be marked and crystals may partially or completely dissolve, this can be useful for confirming, identifying, or characterizing unidentified cells and organisms, particularly bacteria.

II. WBCs in urine sediment
  A. Physiologic processes: A few WBCs may be found in the urine of healthy mammals. Typically, there are fewer than about 5/hpf, but this varies based on the method of urine collection, freshness of sample, centrifuged volume, centrifugation rate and duration, and variable features of individual microscopes.
  B. Analytical concepts
    1. WBCs in urine sediment are enumerated as the range or mean of WBCs seen in most 40–45×-objective fields; a field of view at these magnifications is considered a high-power field (hpf).
    2. WBCs are not routinely differentiated when found in urine. On stained slides prepared from fresh urine, most of the WBCs are found to be neutrophils; macrophages may also be present, eosinophils may occasionally be prominent, and, in rare cases, neoplastic lymphocytes may predominate. Small epithelial cells may look similar to WBCs.
    3. WBCs deteriorate in urine and get more difficult to identify with time; their numbers may diminish.
  C. Pyuria: increased [WBC] (number/hpf) in urine sediment (Table 8.14) (Figure 8.14)
    1. Urinary tract inflammation
      a. Inflammation of mucosal or submucosal tissues or renal parenchyma may cause WBCs (mostly neutrophils and monocytes) to migrate from blood to urine. Inflammation can be caused by infections (by bacteria, fungi, or parasites) or noninfectious processes (neoplasia, urolithiasis, or necrosis).

Table 8.14. Major disorders and conditions that cause abnormal findings in urine sediment – cells and organisms

Pyuria
Urinary tract inflammation:* infectious and noninfectious
Genital tract inflammation
Lymphoma (rare)
Hematuria
Urinary tract hemorrhage:* pathologic (damaged vessels or coagulation defect), iatrogenic
Genital tract hemorrhage: pathologic, estrus
Epithelial cells in sediment
Can be found in low numbers in healthy animals*
Inflammation/hyperplasia in genitourinary tract*
Epithelial neoplasm in genitourinary tract
Bacteriuria
Infection in urinary or genital tract*
In vitro growth or contamination*
Subclinical (occult, asymptomatic) bacteriuria
Other organisms
Fungi, yeast (*Candida* sp., *Blastomyces* sp., *Cryptococcus* sp.) and hyphal forms
Nematode ova or larvae (*Dioctophyma renale*, *Capillaria plica*)
Microfilaria (with hematuria)

* A relatively common disorder, condition, or mechanism

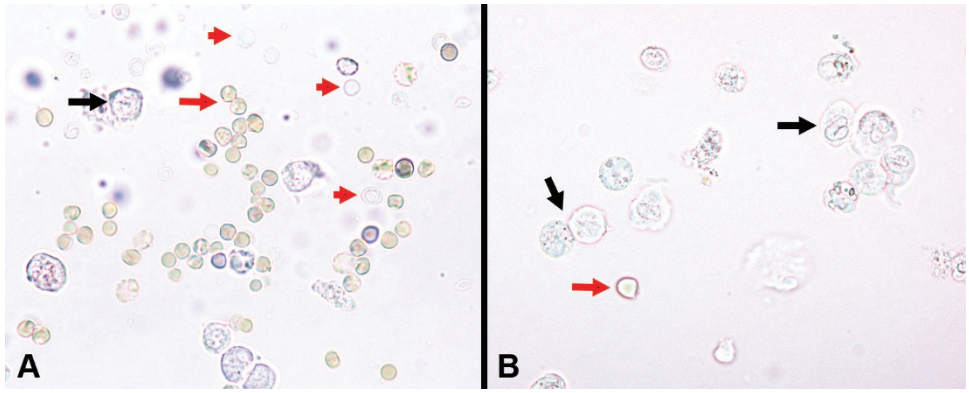


Figure 8.14. WBCs and RBCs in urine sediment. WBCs (*black arrows*) are larger than RBCs (*red arrows*), grainy, and may have an apparent nucleus (*right-most arrow in B*). Hemoglobin imparts a light tan color to RBCs (*long red arrow*) but they are pale ghosts when lysed (*short red arrows*). 20× objective, no stain

b. The method of sample collection (voided, cystocentesis, or catheterization) may help determine the site of the inflammatory process, e.g., WBCs might be from anywhere in the genitourinary tract in a voided specimen, but their presence in a cystocentesis sample indicates the source is somewhere from the kidneys to, and including, the proximal urethra. Other clinical information may also help determine the site of the inflammation, e.g., pollakiuria and stranguria would suggest lower urinary tract involvement, whereas fever, neutrophilia, or azotemia would suggest nephritis. WBC casts indicate inflammation associated with renal tubules.

2. Genital tract inflammation
   a. WBCs may enter urine from the male or female genital tract before (e.g., prostate) or during (e.g., preputial or vaginal source) urination. The chance of contamination during urination is reduced if the sample is collected midway through voiding.
   b. The inflammatory process may or may not be of clinical significance (e.g., mild posthitis).
3. Neoplastic WBCs (Figure 8.15): Rarely, neoplastic WBCs exfoliate into urine from a neoplasm involving the urinary tract (e.g., lymphocytes from lymphoma). Although this is not truly pyuria, these cells may be reported as WBCs (which they are). Atypical features (e.g., large size, nucleoli) may suggest they are not inflammatory WBCs.

III. RBCs in urine sediment (Figures 8.14 and 8.15)
   A. Physiologic processes: A few RBCs (fewer than about 5/hpf) may be found in the urine of healthy mammals.
   B. Analytical concepts
      1. RBCs in urine sediment are enumerated as the range or mean of RBCs seen in most 40–45×-objective fields (hpf).
      2. RBCs may crenate in urine and can (but should not) be confused with WBCs in nonstained sediment. They may swell in hypotonic urine.
      3. RBCs may lyse in urine before or after urine is collected and appear as ghost cells (cell outlines) (Figure 8.14A). Lysis tends to occur in unconcentrated or mildly concentrated urine (USG_{ref} < 1.015) or very alkaline urine, especially if analysis is delayed.

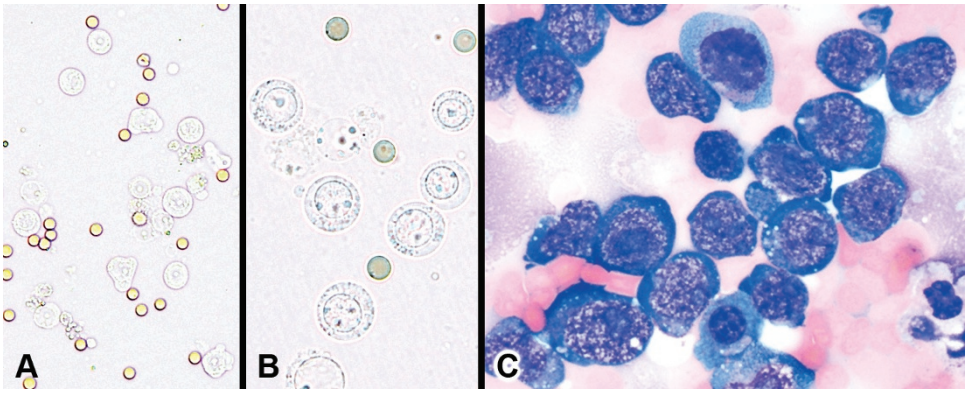


Figure 8.15. Neoplastic lymphocytes in urine sediment. **A and B.** Unstained wet preparation of urine sediment containing RBCs and individual round cells with small amounts of cytoplasm, round nuclei, and nucleoli. 20× (A) and 40× (B) objectives. **C.** Air-dried Wright-stained sediment preparation showing neoplastic lymphocytes and partially lysed RBCs, 50× objective.

4. If RBCs are seen in the urine sediment, the heme reaction should be positive (assessing prior to centrifugation).

C. Hematuria: increased [RBC] (number/hpf) in urine sediment (Table 8.14)
1. Pathologic hemorrhage
   a. Vascular damage caused by trauma, inflammation, renal infarcts, or other processes; common in animals with urinary tract infections
   b. Thrombocytopenia, thrombocytopathia, or von Willebrand disease and the resultant poor repair of small vessels
   c. Coagulopathies (acquired or congenital)
   d. Idiopathic (may be glomerular)
   e. Abnormal RBC shapes have been reported in human patients with glomerular hemorrhage, but differentiation of glomerular and nonglomerular hemorrhage by urine RBC appearance has not been reported in veterinary species.
2. Iatrogenic hemorrhage
   a. Blood vessels may be damaged during bladder palpation, cystocentesis, or catheterization. Voided samples may be used to help differentiate pathologic from iatrogenic hemorrhage.
   b. Animals with hemostasis defects or urinary mucosal disease may be more prone to iatrogenic hemorrhage.
3. Genital tract hemorrhage (associated with estrus) in voided samples

IV. Epithelial cells in urine sediment (there is no word for this) (Figure 8.16)
A. Physiologic processes
1. Epithelial cells are constantly sloughing from the urinary tract mucosa and are replaced by new cells. Thus, epithelial cells are expected in the urine of healthy animals.
2. Types of epithelial cells vary within the urinary tract. Renal tubular epithelial cells are cuboidal in situ but may be round in suspension; transitional epithelial cells lining the mucosa from the renal pelvis through most of the urethra appear round, and squamous epithelial cells from the distal urethra appear round or polygonal (Figure 8.16A–C). In

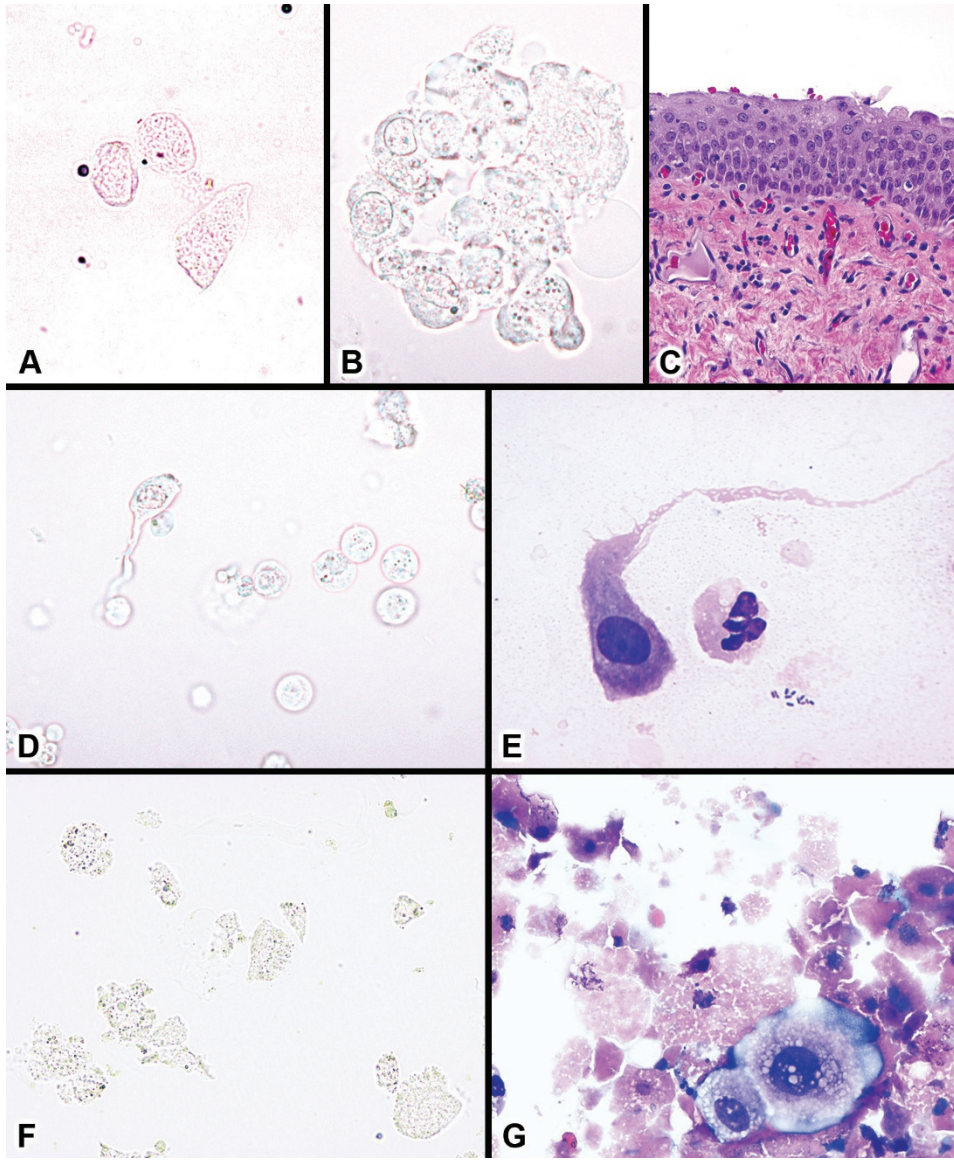


Figure 8.16. Epithelial cells found in urine sediment. **A.** Angular (squamous) and round (squamous or transitional) epithelial cells. **B.** Cluster of epithelial cells (probably transitional) with various amounts of grainy cytoplasm and two round nuclei apparent (*middle-left*). **C.** Healthy bladder wall showing its thickness and increased amounts of cytoplasm in superficial cells compared to basal cells, 20× objective, hematoxylin and eosin stain. **D.** Caudate epithelial cell (*upper left*) and mostly WBCs. **E.** Caudate cell, neutrophil, and cluster of bacterial rods in air-dried, Wright-stained sediment from same sample as D, 100× objective. **F.** Variously sized round to angular grainy particles similar to cell cytoplasm or granular casts (see G). **G.** Air-dried, Wright-stained sediment from same sample as F showing two intact epithelial cells with anisocytosis and anisokaryosis surrounded by necrotic epithelial cells or cell fragments with hypereosinophilic cytoplasm and shrunken or no nuclei, 50× objective. A, B, D, and F: unstained, wet urine sediment, 40× objective.

dogs, caudate epithelial cells (Figure 8.16D and E) were found to line the renal pelves and the ureters; they were not found in the lower urinary tract.[202] Note: Many people have transitioned to using the term *urothelium* for urinary transitional epithelium, despite the fact that the epithelium lining the urinary tract (urothelium) varies and includes cuboidal to columnar cells proximally, squamous epithelial cells distally, and transitional epithelial cells in between.

B. Analytical concepts
   1. Epithelial cells in urine sediment are enumerated as the range or mean of epithelial cells seen in most 10×-objective fields (per low-power field, /lpf).
   2. Some laboratory reports separate epithelial cell populations by origin, but differentiation is problematic:
      a. Round transitional epithelial cells are difficult or impossible to differentiate from the round cells that occur in the intermediate and even more superficial layers of squamous epithelium.
      b. In nonstained sediment, individual renal tubular epithelial cells are difficult or impossible to differentiate from macrophages or small transitional epithelial cells, and clumps of renal tubular epithelium may look similar to transitional epithelium.
   3. All cells deteriorate in urine and thus are best examined in fresh urine. Deteriorated epithelial cells, like necrotic epithelial cells (Figure 8.16F and G), may be difficult to identify.

C. Clinical significance of epithelial cells in urine (Table 8.14)
   1. Epithelial cells may be found in the urine of healthy animals, especially in catheterized samples.
   2. More transitional epithelial cells (urothelium) may slough from inflamed or hyperplastic mucosa.
   3. Neoplastic epithelial cells may be detected in urine sediment. Differentiation of malignant from nonmalignant cells is better accomplished by microscopic examination of stained, air-dried preparations of cells collected by traumatic catheterization or needle biopsy, but preparations of centrifuged, fresh, newly formed urine may be useful. Such assessment is particularly indicated when many cells and cell clusters (Figure 8.16B) or sheets are present. Individual and clustered dysplastic and reactive cells may be present in urine from animals with cystitis and reactive hyperplasia.

V. Bacteria in urine sediment

A. Physiologic processes: Urine formed by the kidneys should be sterile. Low numbers of bacteria may access the urine via the distal urinary tract or genital tissues.

B. Analytical concepts
   1. Bacteria (especially cocci) may be difficult or impossible to differentiate from small particulate debris by routine sediment examination. Rods or chains of cocci are more easily identified with certainty than are single cocci or clusters of cocci (Figure 8.17A–D).
   2. Bacteria may multiply in urine after it is collected.
   3. There is no standard method of reporting the concentration of bacteria in urine sediment. Bacteria may be reported as the relative density (from few to many) of bacteria seen in most 40–45×-objective fields (hpf) or bacteria may simply be reported as present or absent. Routine centrifugation of urine may not concentrate bacteria appreciably in the sediment because they may not "spin down" with the forces that are used.[203,204] This is in contrast to other suspended particulates such as cells.

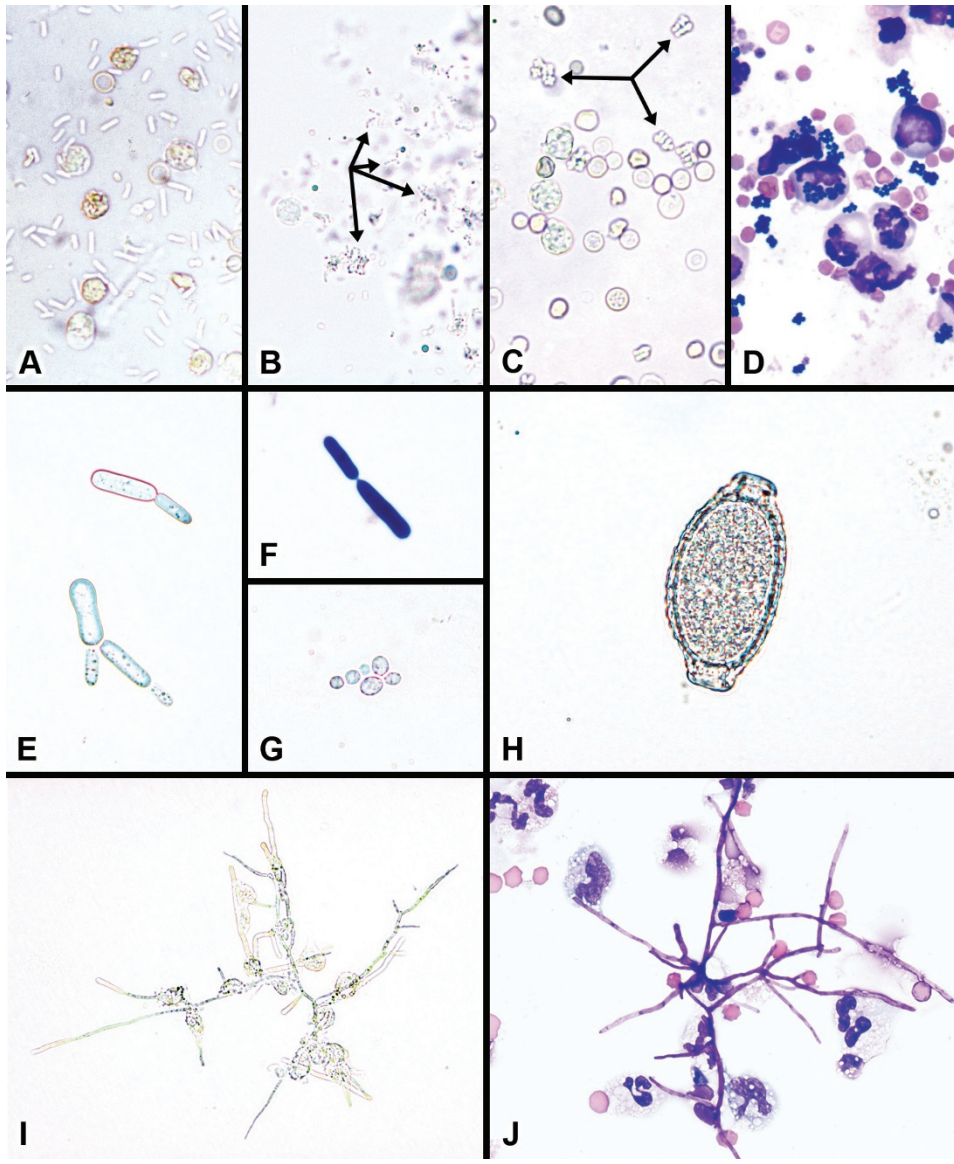


Figure 8.17. Organisms found in urine sediment. **A.** Large bacterial rods with WBCs and RBCs (courtesy of Don Schmidt, University of Missouri). **B.** Small bacterial cocci forming chains (*arrows*). **C.** Clusters of cocci (see D) with WBCs and RBCs. **D.** Air-dried Wright-stained sediment (same sample as C) showing neutrophils, RBCs, and clusters of bacterial cocci (blue) found extracellularly and within neutrophils. **E.** Elongate budding yeast, possibly *Cyniclomyces guttulatus*. **F.** Budding yeast in air-dried Wright-stained sediment preparation (same sample as E). **G.** Round to ovoid budding yeast. **H.** Ovum of *Pearsonema*. **I.** Branching fungal hyphae with adherent cells. **J.** Air-dried, Wright-stained sediment (same sample as I) showing branching hyphae with RBCs, neutrophils, and macrophages, 50× objective. A, B, C, E, G, H, and I: unstained, wet urine sediment, 40× objective.

4. Stained air-dried urine preparations for microscopic examination may be more helpful for detecting bacteriuria than routine wet-mount preparations.[205]

C. Bacteriuria (Table 8.14)
1. Determining the clinical significance of bacteriuria involves consideration of the presence or absence of pyuria, the source of the sample, the concentration of bacteria, other evidence of a urinary tract infection, and concurrent disorders.
2. The absence of detectable bacteria in urine sediment does not exclude the possibility of an infection. One should use quantitative urine culture methods whenever a urinary tract infection is suspected. As many as 10,000/mL (rods) or 100,000/mL (cocci) may be required for detection by routine UA, but as few as 1000/mL may be significant in a cystocentesis sample. The decision threshold (based on quantitative culture) between significant bacteriuria and contamination depends on the species of animal and the urine collection method used.[206]
3. Bacteriuria without clinical signs, either with pyuria or without pyuria, occurs in a low percentage of healthy dogs, cats, people, and likely other species, and in a higher percentage of individuals with certain disorders (e.g., diabetes mellitus). Treatment of this subclinical bacteriuria is probably contraindicated except in rare populations or individuals.[207]

VI. Organisms other than bacteria (Table 8.14)
A. Yeast (e.g., *Candida*, *Blastomyces*) (Figure 8.17E–G), hyphae (e.g., *Aspergillus* and other genera including various contaminants) (Figure 8.17I and J), algae (*Prototheca*), and parasitic structures (e.g., *Dioctophyma* ova, *Pearsonema* (*Capillaria*) ova or larvae, *Stephanurus* ova, and *Dirofilaria* microfilaria) can be found in urine (Table 8.14). Identification of fungal structures may require culture.
B. Parasitic structures may be present because of sample contamination with fecal material. Ova of urinary *Pearsonema* (*Capillaria*) species are similar to ova of *Trichuris* species, but they tend to have offset polar plugs (Figure 8.17H).

VII. Casts in urine sediment
A. Physiologic processes
1. Casts are cylindrical concretions with round, square, irregular, or tapered ends and varying width that mirrors the tubular segments in which they formed. They are formed in renal tubular lumens from proteins, intact cells, or deteriorated cellular material. Uromodulin (Tamm–Horsfall mucoprotein) secreted by epithelial cells of the thick ascending limb of the LoH (mostly) and DCT provides the matrix for hyaline and most other casts. Uromodulin aggregation into casts is promoted by acidic pH and more concentrated urine, the latter because of greater uromodulin and electrolyte concentrations.[208]
2. A few casts other than hyaline casts may form during the normal sloughing of tubular epithelial cells that occurs daily. Casts seen in healthy mammals typically are hyaline casts or fine granular casts. Typically, only a few hyaline or granular casts are found (< 3/lpf), but a shower of casts may occur after physical activity or rehydration.

B. Analytical concepts
1. Casts in urine sediment are enumerated as the range or mean of casts seen in most 10×-objective fields (lpf).
2. Casts are classified by their appearance, which reflects their content: fine and coarse granular (cell debris and plasma protein), epithelial cell (tubular epithelial cells), RBC,

WBC, fatty (lipid droplets or lipid accumulation within deteriorated cells), waxy (perhaps deterioration of a granular cast), or mixed (combinations of the previously mentioned types).

3. Casts deteriorate in urine (especially alkaline urine)[208] and thus are best detected in fresh urine samples.
4. Casts may be pigmented by bilirubin (yellow), hemoglobin (red to brown), or myoglobin (red to brown).

C. Cylindruria (Figure 8.18 and Table 8.15)

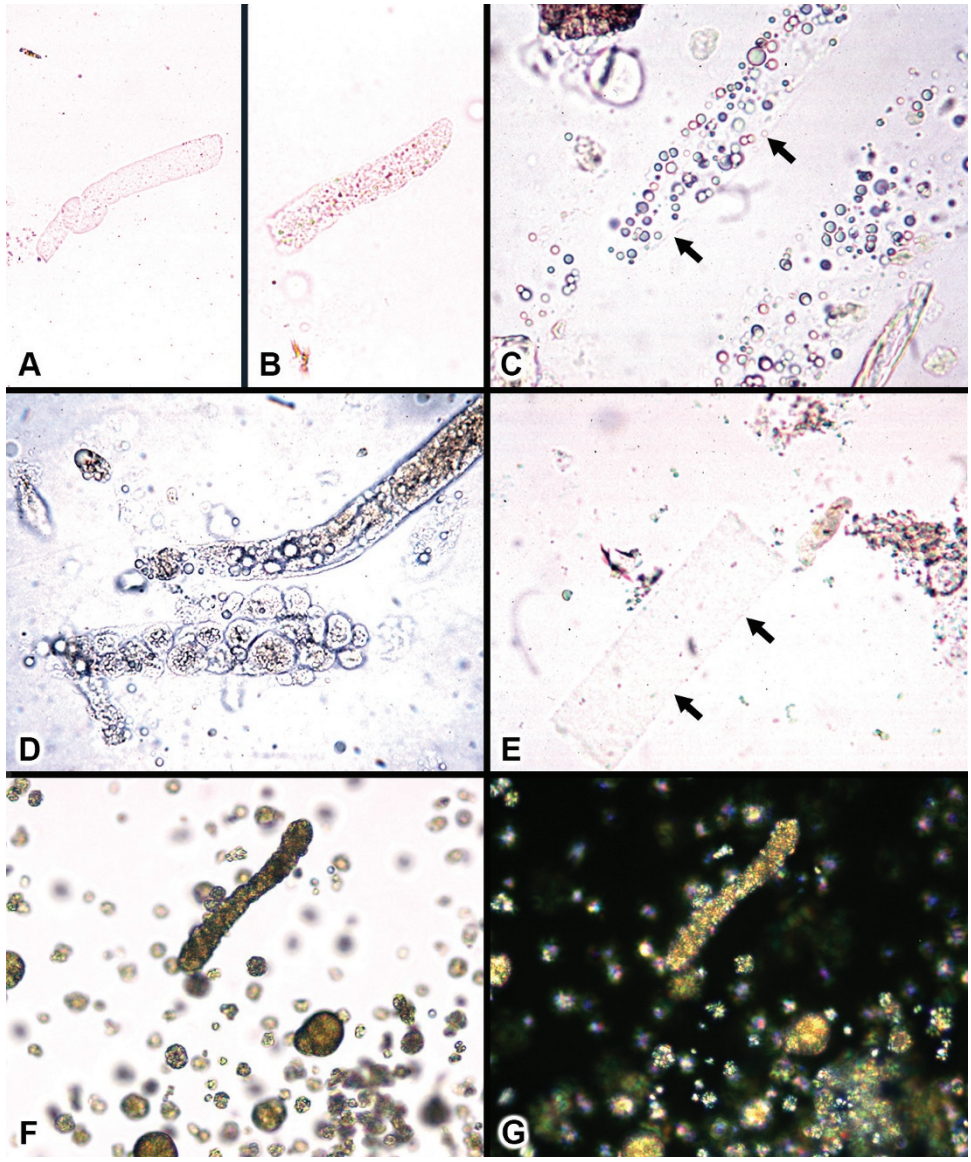


Figure 8.18. Casts found in urine sediment. **A.** Hyaline cast (10× objective). **B.** Granular cast. **C.** Lipid-containing casts. **D.** Epithelial cell casts. **E.** Waxy cast. **F and G.** Crystal cast and crystals of melamine cyanurate shown without (F) and with (G) use of a polarizing filter. Wet urine sediment, 40× objective (except A).

1. *Cylindruria* (not cylinduria) is the presence of casts in urine. Casts form in renal tubules but may not be flushed into urine, or they may be discharged intermittently in showers. Therefore, the absence of cylindruria does not exclude active renal tubular disease.
2. Hyaline casts (Figure 8.18A) may be found in healthy animals and occur more commonly in animals with glomerular proteinuria.

Table 8.15. Major disorders and conditions that cause abnormal findings in urine sediment – casts and crystals

Cylindruria
Can be found in low numbers in healthy animals (hyaline, granular)
Associated with glomerular proteinuria (especially hyaline casts)
Active renal tubular cell degeneration, inflammation, or hemorrhage: nephrosis, nephritis
Crystalluria (urine pH in which crystals tend to occur)
Ammonium (bi)urate (pH usually $\leq$ 7): seen in healthy mammals; common in Dalmatian dogs and English bulldogs; suggest liver dysfunction, portosystemic shunts, or hepatic microvascular dysplasia in dogs and cats
Bilirubin (pH < 7):* seen with bilirubinuria
Calcium carbonate (pH $\geq$ 7):* healthy herbivores; not seen in dogs and cats
Calcium oxalate dihydrate (pH usually $\leq$ 7):* seen in healthy mammals, suggest possible hypercalciuria or hyperoxaluria (including ethylene glycol toxicosis or ingestion of oxalate-rich plants such as *Halogeton* and *Amaranthus* in ruminants)
Calcium oxalate monohydrate (pH usually $\leq$ 7): suggest possible hypercalciuria or hyperoxaluria, especially ethylene glycol toxicosis, also chocolate toxicosis; hemp seed variant common in horses
Calcium phosphate (pH usually $\geq$ 7): seen in healthy dogs, dogs with calcium phosphate uroliths, or dogs with persistent alkalinuria
Cholesterol (pH $\leq$ 7): uncommon, but may be seen in healthy dogs; suggest tissue necrosis or hypercholesterolemia and proteinuria as seen with protein-losing nephropathy
Cystine (pH usually $\leq$ 7): seen with defective tubular resorption of amino acids (cystinuria)
Drug crystals (sulfa, ampicillin, contrast media, primidone): reflect presence of compound in urine
Leucine (pH < 7): rare; suggest liver disease
Struvite (magnesium ammonium phosphate)* (pH usually $\geq$ 7): common in dogs and cats with alkalinuria, may be associated with urease-producing bacteria
Tyrosine (pH < 7): rare; suggest liver disease
Urate, sodium (pH $\leq$ 7) or NH_4^+ (pH variable): seen in healthy mammals; common in Dalmatians and English bulldogs; suggests liver dysfunction, portosystemic shunts, or hepatic microvascular dysplasia in dogs and cats
Uric acid (pH < 7): similar significance as urates; has occurred after chemotherapy for lymphoma in a dog
Xanthine (pH $\leq$ 7): rare; treatment with xanthine oxidase inhibitors (allopurinol) or a metabolic defect (hereditary xanthinuria)

* A relatively common disorder, condition, or mechanism

a. Attachment of uromodulin to tubular epithelial cells is essential for hyaline cast formation. Without attachment, protein aggregates are flushed from the kidney.[209]
b. Hyaline casts from healthy people contained exclusively uromodulin whereas the hyaline casts from people with glomerulonephritis also contained fibrin, immunoglobulins, and complement.[210]

3. Epithelial or fatty casts (Figure 8.18C and D) typically reflect active tubular degeneration or necrosis. They may be evidence of toxic nephrosis (e.g., gentamicin induced, or hemoglobinuric and myoglobinuric nephropathies) or renal ischemia. In one study, the epithelial casts consisted of epithelial cells adhered to hyaline casts.[209]
4. Granular casts (Figure 8.18B) may reflect tubular degeneration, necrosis, or inflammation. They may also form from the precipitation of plasma proteins. They can be found in the urine of healthy mammals, possibly related to normal cell turnover or to entrapped proteins. Via bright-field, phase, and electron microscopy, four types of granular casts were identified, their composition varying with the associated pathologic state.[211] Contents included degraded WBCs, degraded epithelial cells, paraproteins, or bacteria.
5. WBC casts reflect inflammation involving renal tubules (e.g., with tubular nephrosis or pyelonephritis).
6. RBC casts reflect glomerular or tubular hemorrhage.
7. Waxy casts (Figure 8.18E) are uncommon and seen primarily with chronic renal disease.
8. Hemoglobin and myoglobin casts are red- to brown-pigmented, granular casts and may be seen with hemoglobinuria or myoglobinuria.
9. Casts containing many crystals may be called crystal casts or crystalline casts (Figure 8.18F and G). They are uncommon and may be reported as granular casts. They indicate crystal formation within tubules, as may occur with acute crystalluric nephropathies, including those involving oxalates, urates, or melamine cyanurate.

D. Pathogenesis (prevailing theories)
1. Hyaline casts form from the conglutination of the uromodulin that is secreted mostly by tubular cells of the thick ascending limb of the LoH. The reason for the conglutination is not understood, but it is enhanced by higher concentrations of uromodulin and ions.[212]
2. Granular, lipid, and cellular casts form when cellular debris or cells are trapped in or adhere to uromodulin. Granular casts may also be formed by the incorporation of plasma proteins into the uromodulin matrix. Waxy casts may be formed by the deterioration and solidification of granular casts or may represent a condensed from of a hyaline cast.[209]

VIII. Crystals
A. Physiologic concepts
1. Crystals represent the precipitation of salts (cation + anion).
2. Some salts tend to form at alkaline pH but dissolve at acidic pH, whereas others tend to form at acidic pH but dissolve at alkaline pH. A salt that is soluble at a certain pH may form crystals if the concentration of the ions is high enough (saturation point),

the materials are present to begin crystal formation (nucleation), and the balance of promoters to inhibitors of nucleation and crystal growth favor crystal formation.

B. Analytical concepts
   1. Crystals can vary markedly in size but are enumerated as the number of crystals seen in most 10×-objective fields (lpf).
   2. Enhanced crystal formation may occur in stored urine because of evaporation or because of changes in temperature (especially refrigeration) or pH.[105] Conversely, in other stored samples, some crystals may dissolve.
   3. Crystals are routinely identified by their microscopic appearance (Figure 8.19), although many crystals have multiple forms, some of which are uncommon. Chemical and crystallographic techniques can be used for further characterization (see the Urolith Analysis section).

C. Crystalluria (Table 8.15)
   1. The degree of crystalluria depends on the pH, concentration of ions, and temperature of the urine.
   2. Most types of urine crystals can be found in healthy animals. However, increased numbers in the urine sediment may suggest the presence of certain pathologic states that are causing changes in either urine pH or ion concentration.
   3. Presence or absence of crystalluria is not a reliable indicator for the presence or absence of urolithiasis (see the Urolith Analysis section), but crystalluria is a risk factor for urolithiasis and may be monitored during medical management of urolithiasis.

IX. Other sediment findings that are of little or no diagnostic significance

A. Lipid droplets (Figure 8.20A)
   1. Probably from renal tubular epithelium
   2. Most common and abundant in cats, which normally store triglycerides in renal tubular cells
   3. May represent contaminating lubricants used during sample collection
   4. Differentiated from RBCs and yeast by consistent round appearance in a layer above other nonsoluble material including cells, crystals, and casts

B. Oval fat bodies (Figure 8.20B): Oval fat bodies typically are lipid-containing renal tubular epithelial cells or macrophages, but they may also reflect a prostatic source.[213]

C. Mucus strands represent urogenital secretions and are abundant in urine of horses.

D. Spermatozoa (Figure 8.20C)
   1. Spermatozoa are an expected finding in urine from intact male animals, especially in voided samples, but also in cystocentesis samples.
   2. Occasionally, spermatozoa are found in urine of female animals after breeding.

EXAMINATION OF SUSPENDED ELEMENTS BY OTHER METHODS

I. Examination of uncentrifuged urine

A. Because of the time required to prepare urine for a sediment examination and perhaps because of variations in results due to different centrifuged volumes or centrifugation forces, there have been efforts to assess if centrifugation is needed to detect abnormalities in the urine.

B. In a published study of 167 human urine samples assessed with and without centrifugation, WBCs, RBCs, and casts (hyaline and granular) were found in more centrifuged samples than in uncentrifuged samples.[214] Bacteria were found in all

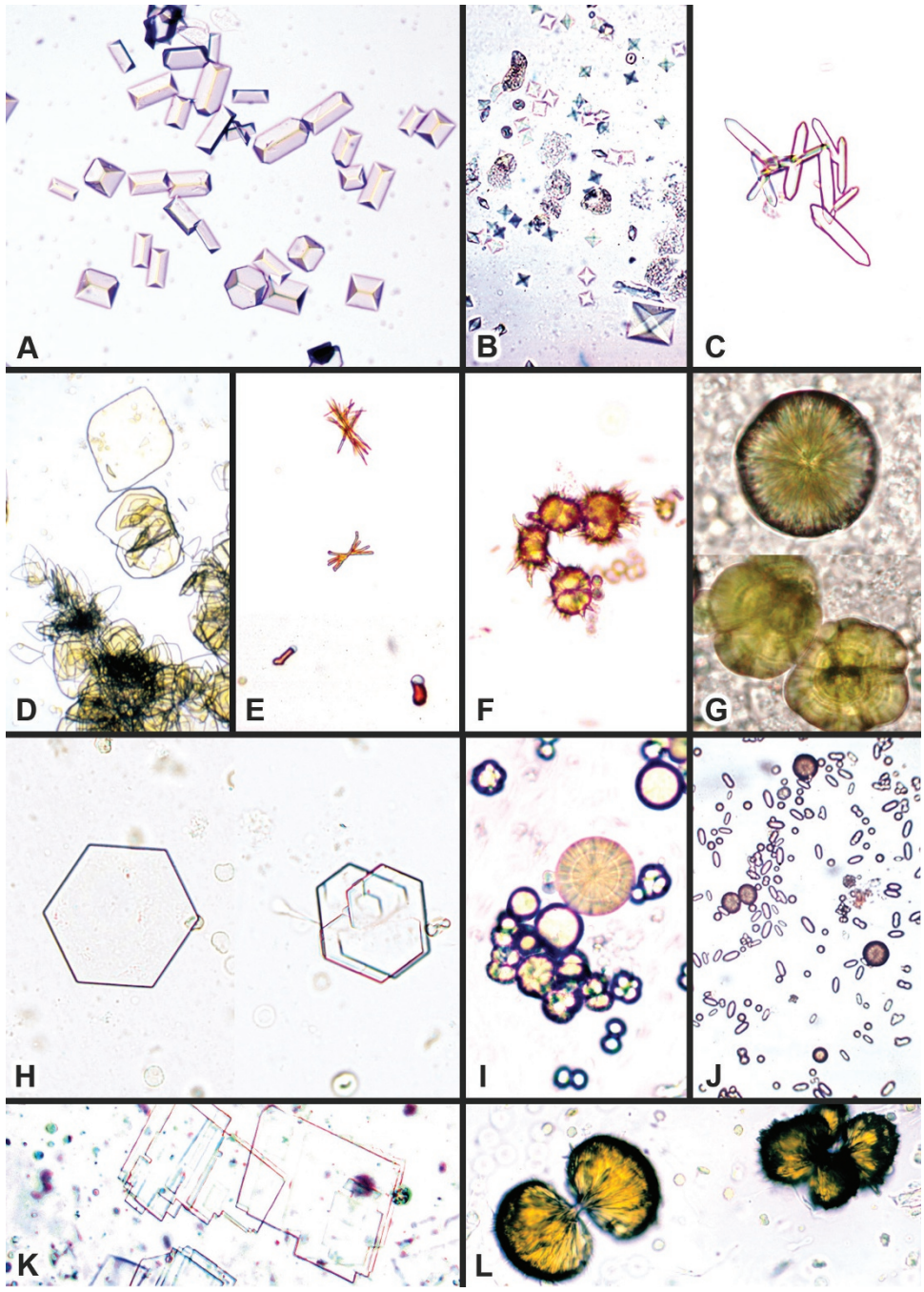


Figure 8.19. Crystals found in urine sediment. **A.** Struvite crystals, typical form. **B.** Calcium oxalate dihydrate crystals. **C.** Calcium oxalate monohydrate crystals, picket fence or canoe form. **D.** Uric acid crystals. **E.** Bilirubin crystals, including pointed, blunt, and flashlight forms. **F.** Ammonium biurate crystals, thornapple form. **G.** Melamine cyanurate crystals. **H.** Cystine crystals; note sperm, emphasizing that some cystinuria is sex-linked. **I and J.** Calcium carbonate crystals, mixed forms (horse). **K.** Cholesterol crystals (courtesy of Don Schmidt). **L.** Sulfa crystals and RBCs (courtesy of Don Schmidt). Wet urine sediment, 40× objective.

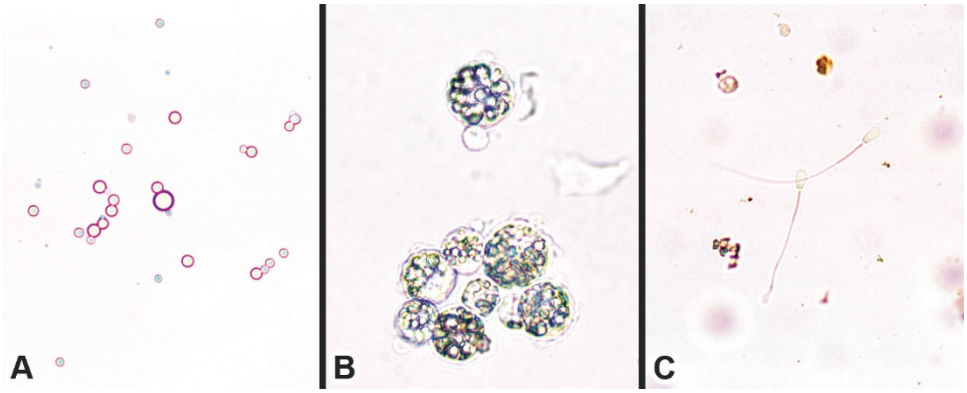


Figure 8.20. Miscellaneous urine sediment findings. **A.** Lipid droplets with distinct outlines and varied size; they float above sedimented formed elements. **B.** Oval fat bodies, i.e., lipid-containing epithelial cells or macrophages. **C.** Sperm. Wet urine sediment, 40× objective.

centrifuged samples but in only 159 of the uncentrifuged samples. When present, bacteria and WBCs were present at greater number per field in the centrifuged samples.

C. Using urine sediment quality-control materials and fresh canine RBCs rather than actual urine, recovery of fixed cells (WBCs) and RBCs was poor and low compared to the traditional method (with centrifugation) when well-mixed samples were pipetted directly onto slides without prior centrifugation.[199]

D. Gravimetric sedimentation of 60 µL of urine sediment quality-control material (fixed cells) or saline suspensions of RBCs in microtiter plate wells yielded similar cell recoveries as a traditional method that used centrifugation and decanting, and with greater precision.[199] However, the method required an inverted microscope and neither actual urine samples nor recovery of microorganisms were assessed.

II. Examination by automated methods

A. Automated methods using flow cytometry or image analysis of cells in either uncentrifuged or sedimented urine have been developed. Compared to routine microscopic methods, these automated methods have less labor costs and are more reproducible.

B. Table-top urine sediment analyzers have been developed for veterinary use. Samples must be gently mixed before analysis to distribute formed elements evenly in the small sample volumes that are analyzed. Formed elements are counted, partially differentiated, and reported quantitatively (/hpf or /µL) or semiquantitatively in the following limited categories, with images available for confirmation or further characterization:

1. Cells: RBCs, WBCs, squamous epithelial cells, other epithelial cells
2. Bacteria: rods, cocci
3. Casts: hyaline, nonhyaline
4. Crystals: struvite, calcium oxalate dihydrate, other or unclassified

C. SediVue and VETSCAN urine sediment analyzers were assessed under field conditions by using urine quality-control materials to determine if the analyzers met analytical performance claims for detection of RBCs, WBCs, cystine crystals, and bacteria.[215] One performed better than the other, but deficiencies included over-detection of bacteria, under-detection of crystals, and a few false-negative results for RBCs or WBCs.

D. Several studies assessing the performance of the SediVue or Anvajo Vet Fluidlab 1 urine sediment analyzer for use with clinical canine and feline samples have been published. These have included assessments of RBCs, WBCs, epithelial cells, struvite crystals, calcium oxalate dihydrate crystals, bacteria, and casts. Results are difficult to interpret because of lack of a gold standard UA method, use of different comparison methods with unknown applicability to methods used by others, inconsistent urine volumes for comparison methods, lack of well-established decision limits for cell concentrations, variations in analyzer software versions, and more. However, studies generally indicate that current veterinary automated urine sediment analyzers (i) can usually detect clinically relevant concentrations of RBCs and WBCs, (ii) have significant limitations in the reliable detection of other formed elements, and (iii) should not be used without evaluation of captured images by trained individuals.

QUANTITATIVE URINALYSIS

I. Basic concepts

A. Quantitative UA includes procedures in which chemical or physical properties of urine are measured with quantitative assays. Assays used for routine UA are qualitative or semiquantitative. The purpose of a quantitative analysis is typically to quantify the amount of a substance that is excreted by the kidneys.

B. Urine analyte concentrations can be difficult to interpret because concentrations depend on two major variables: (i) the amount of analyte excreted per unit time and (ii) the amount of H_2O excreted per unit time. The relationship of these variables is expressed in Eq. 8.4 in the context of amounts excreted per day. Note that an animal would excrete the same amount of analyte per day if there were a doubling of the analyte concentration but a halving of the urine volume. An analyte's urine concentration can be assessed by considering the USG_{ref}, because changes in USG_{ref} typically are inversely related to changes in urine volume (except in renal failure). More precise assessments usually are accomplished by either timed urine collections or by calculating clearance ratios.

C. Examples of analytes whose renal excretion is assessed via quantitative assays

1. Electrolytes: Na^+, K^+, Cl^-, Ca^{2+}, and PO_4
2. Protein: usually total protein, also albumin, occasionally other individual proteins
3. All solutes combined: total solute excretion via urinary system assessed by osmolality

D. Assay methods used to quantify the analyte concentration in serum may or may not accurately measure an analyte concentration in urine. This is illustrated by the following examples.

1. Urine [Crt]: To measure urine [Crt], the urine sample needs to be diluted to get the [Crt] into the assay's analytical range. This process can cause inaccuracies.[216] NonCrt chromogens may give positive interference with some Crt assays, although kinetic Crt assays are designed to minimize the interference of nonCrt chromogens.
2. Urine [tCa^{2+}]: Unless there is proteinuria, urine Ca^{2+} exists as free ions, as ions complexed with anions, and possibly within crystals. To measure urine [tCa^{2+}], most calcium methods specify that the urine sample should be acidified before analysis so that Ca^{2+} is in a soluble form and thus able to react with reagents. However, acidification of human urine does not appear necessary other than for samples from patients with crystalluria and uroliths.

a. Small, statistically significant differences were found in urine [Ca^{2+}] between acidified and nonacidified samples when urine contained amorphous, calcium oxalate, or struvite crystals and when measured with a cresolphthalein complexone assay; differences were unlikely to affect clinical decisions.[217] In another study using the same assay, significant differences were not found in urine [Ca^{2+}] between acidified and nonacidified samples, but the presence or absence of crystalluria was not determined.[218]

b. Using a different assay to measure [tCa^{2+}] in 567 urine samples including 46 from patients with hypercalciuria (but with no information regarding crystalluria), there was no consistent effect of acidification on urinary [tCa^{2+}].[219] After acidification, three hypercalciuric patients were reclassified as normocalciuric (acidification decreased the [tCa^{2+}]).

3. Urine [K^+]: Equine, bovine, and feline urine appear to contain a substance that inhibits the reaction of K^+ with ion-selective electrodes. In equine urine samples with [K^+] from 100–450 mmol/L determined by flame photometry, the indirect potentiometry method did not measure a [K^+] above 100 mmol/L. Similar results were found in bovine and ovine urine samples. The feline data indicated a definite proportional bias with [K^+] up to about 320 mmol/L. There was good assay agreement in canine urine, but [K^+] did not exceed 125 mmol/L. Across species, there was good agreement in nearly all urine samples when the [K^+] was < 50 mmol/L.[220]

E. The following factors may need to be considered when interpreting quantitative UA results:
1. Are there appropriate RIs for the patient? For some analytes, body weight, diet, age-related factors, or treatments can alter urinary excretion of substances.
2. Is an analyte's urinary excretion decreased because the kidneys are conserving the analyte or because less analyte is being presented to the kidneys?

II. 24-h excretion studies

A. This is the most definitive method of determining urinary excretion of an analyte, but it is not frequently used in veterinary medicine because of several factors. First, it requires complete and timed urine collection that is difficult in most domestic mammals. Second, RIs are generally not available for each species, for different assay methods, and for different diets.

B. Procedure: Begin the urine collection period by completely emptying the urinary bladder and discarding the urine. Collect all urine that is produced in 24 h. End the collection period by emptying the bladder. Measure the total volume of urine collected in 24 h. Measure the analyte's concentration in the collected urine. Finally, calculate the quantity of substance excreted (e.g., Eq. 8.5a). Because of the marked variation in the sizes of domestic mammals, results are usually expressed per kilogram of body weight (Eq. 8.5b).

Amount of analyte excreted/d = analyte concentration × urine volume/d

Example: analyte concentration = 50 mg/dL; urine volume/d = 300 mL/d (**8.5a**)

Amount of analyte excreted/d = 50 mg/dL × 300 mL/d = 150 mg/d

Amount of analyte excreted/kg/d = analyte concentration × urine volume/d ÷ kg

Example: same analyte concentration and urine volume; body weight = 5 kg (**8.5b**)

Amount of analyte excreted/kg/d = 150 mg/d ÷ 5 kg = 30 mg/kg/d

C. Because a 24-h excretion study is difficult, more convenient methods to assess urinary excretion are usually used. These include analyte urine-to-plasma ratios or analyte-to-Crt ratios. Although more convenient, they may be less accurate.

III. Analyte urine-to-plasma ratios

A. Theory

1. The rate of urinary excretion or clearance of a substance can be calculated by using an excretion rate formula (Eq. 8.6a).

$$\text{Analyte excretion rate} = \frac{[\text{Analyte}]_u}{[\text{Analyte}]_p} \times \text{volume}_u \div \text{time} \div \text{body weight} \quad (\mathbf{8.6a})$$

$$\text{Analyte excretion rate} \propto \frac{[\text{Analyte}]_u}{[\text{Analyte}]_p} \quad (\mathbf{8.6b})$$

2. If urine volume, time, and body weight are considered constants for a given animal, then the urine-to-plasma ratio is proportional to the rate of urinary excretion of a substance (Eq. 8.6b). Those factors are not constants, but the ratio does tend to reflect the urinary excretion of a substance (analyte) or total solutes.

B. Analyte urine-to-plasma ratios for differentiation of prerenal azotemia and renal azotemia

1. The urine osmolality to plasma osmolality ratio reflects the kidneys' ability to conserve H_2O.
   a. A high ratio reflects an ability to concentrate solutes. An animal with prerenal azotemia caused by dehydration should have a high ratio.
   b. A ratio near 1.0 indicates isosthenuria and thus failure to concentrate or dilute the filtrate solutes. In an animal with renal azotemia, the ratio is expected to be near 1.0.
   c. A ratio much below 1.0 is not expected in most azotemic animals because the ratio would indicate renal diluting ability is present. Azotemic animals usually either form hypersthenuric urine (a high ratio in prerenal azotemia) or cannot concentrate or dilute their urine (a ratio near 1.0 in renal azotemia).
2. The urine UN to plasma UN ratio and urine Crt to plasma Crt ratio reflect the renal ability to excrete nitrogenous wastes.
   a. Higher ratios reflect the kidneys' ability to excrete nitrogenous waste via urine and thus are evidence of adequate renal function.
   b. Lower ratios reflect decreased renal excretion of urea or Crt (i.e., a decreased GFR) and would support the conclusion of renal azotemia, but they also may occur with prerenal and postrenal azotemias.
3. Published analyte urine-to-plasma ratios for horses illustrate concepts of renal function (Table 8.16). The ratios would vary among species, but the general concepts apply across species.
   a. UN ratio: With prerenal azotemia, the ratio tends to be lower than for healthy horses because less urea is being excreted (decreased GFR causes less urea to enter tubules and there is increased tubular resorption of urea) and $[UN]_p$ increases; even with less urea being excreted, renal conservation of H_2O keeps $[UN]_u$ relatively high. The UN ratio is lower with renal azotemia than with prerenal azotemia because there is less renal conservation of H_2O and thus $[UN]_u$ is not as high as in prerenal samples; also, the $[UN]_p$ tends to be greater in renal than in prerenal states.

Table 8.16. Urinary indices for differentiation of prerenal and renal azotemia in horses

	Healthy horses	Prerenal azotemia	Renal azotemia
Number of horses	6	6	10
$Osmolality_u$ (mmol/kg)	727–1456[d]	458–961	226–495
$Osm_u:Osm_p$[a] ratio	2.5–5.2[d]	1.7–3.4	0.8–1.7
$[UN]_u:[UN]_p$[b] ratio	34–100	15–44	2–14
$[Crt]_u:[Crt]_p$[c] ratio	2–344	51–242	3–37
FE of Na^+ (%)	0.0–0.70	0.0–0.5	0.8–10.1

Source: adapted from Grossman, et al.[221]

[a] Urine osmolality to plasma osmolality

[b] Urine concentration of UN to plasma concentration of UN

[c] Urine concentration of Crt to plasma concentration of Crt

[d] The authors did not explain why some of the healthy horses had greater urine osmolalities and $Osm_u:Osm_p$ ratios. Two explanations seem possible: (i) some of the healthy horses had subclinical dehydration, or (ii) some horses in the prerenal-azotemia group had nonrenal reasons for limited renal concentrating ability.

b. Crt ratio: With prerenal azotemia, the ratio tends to be similar to that of healthy horses because less Crt is being excreted (decreased GFR causes less Crt to enter tubules) but renal conservation of H_2O keeps $[Crt]_u$ relatively high; Crt is not resorbed by renal tubules so $[Crt]_p$ is not increased as much as $[UN]_p$. The Crt ratio is lower with renal azotemia than with prerenal azotemia because there is less renal conservation of H_2O and thus $[Crt]_u$ is not as high as in the prerenal samples; also, $[Crt]_p$ tends to be greater in renal than in prerenal states.

IV. Quantitative urine total protein assays

A. These are typically not part of a routine UA but are used to determine urine [protein] for UPC ratios, for urine electrophoresis calculations, or for 24-h excretion studies.

B. The protein assays should be completed on the supernatant of centrifuged urine because the measured concentrations are 15–50 % greater in uncentrifuged urine, likely because of turbidity and matrix effects caused by cells, crystals, or casts.[222]

C. Analytical principles of clinical assays for measuring concentrations of urinary protein (often called microprotein because of the low concentrations, not because the proteins are small) differ from each other, and the differences can produce analytical biases that may affect appropriate RIs and interpretation of results (Table 8.17). For example, the measured concentrations of a Coomassie brilliant blue assay were about 60 % of the concentrations measured with a pyrogallol red molybdate assay.[224] In addition to issues of analytical specificity, the choice of protein (or proteins) used for the calibrator (standard) solution can lead to different results (e.g., human albumin versus bovine albumin calibrators).[228,229]

V. Urine protein-to-creatinine (UPC) ratio

A. Theory

1. Increased protein loss via the urinary system is best determined by a 24-h protein excretion study, i.e., determining the milligrams of protein lost per day per kilogram of body weight. However, such a study requires a timed and complete urine collection.
2. Crt clearance via the urinary system is considered to be relatively constant in healthy, hydrated individuals. If urinary Crt clearance is decreased, then the rate of glomerular

Table 8.17. Urine protein methods and factors that contribute to quantitative differences

Method	Comments
Turbidimetry[a]	
Benzalkonium chloride	Does not react with hemoglobin[223]
Benzethonium chloride	Reacts similarly with albumin and globulins; reacts with hemoglobin[223]
SSA	Measures lower concentrations than Coomassie brilliant blue when tubular proteinuria is present[224]
Trichloroacetic acid	Less affected by albumin:globulin ratio than the SSA method; must be completed at 20–25 °C[225]
Spectrophotometry[b]	
Biuret assay	Modified version of serum total protein assay
Coomassie brilliant blue	Minimally affected by albumin:globulin ratio; detects immunoglobulin light chains but underestimates their concentration[175]
Coomassie brilliant blue + sodium dodecyl sulfate	Modification provides better quantitation of immunoglobulin light chains, decreases measured concentrations of glomerular proteinuria, and increases measured concentrations of tubular proteinuria[174]
Pyrogallol red molybdate dye + sodium dodecyl sulfate	Minimally affected by albumin:globulin ratio; detects immunoglobulin light chains better than turbidimetric or most other dye assays[176]
Pyrocatechol violet dye	Yields falsely low concentrations when globulins are present but has yieldedsimilar results to pyrogallol red method for light chain proteinuria;[226] overestimates protein concentrations when urine is dilute[227]
Trichloroacetic acid/Ponceau S[c]	More accurate at lower protein concentrations than benzethonium chloride, pyrogallol red, and pyrocatechol violet assays[228]

[a] In turbidimetric assays, acid denatures protein and creates a precipitate that interferes with light transmission.
[b] In spectrophotometric assays, dyes bind to proteins to create a unique color that is measured by increased light absorbance within a defined wavelength.
[c] Dye binding is assessed after protein precipitation with acid.

protein loss should be decreased because Crt passes more easily through the GFB than do protein molecules. However, if more protein enters the urine through damaged glomeruli or through other processes, then the rate of protein excretion compared to Crt excretion will be increased.

3. Considering the aforementioned concepts, comparing the rate of urinary protein loss to the Crt excretion should reflect true changes in protein loss via the urinary system. This comparison reduces the variability caused by variations in the amount of H_2O excreted by kidneys. If all else is equal, renal conservation of H_2O causes a proportional increase in urine [protein] and urine [Crt]. This concept can be seen in the comparison of urinary

excretion formulas and the derivation of the UPC ratio (Eq. 8.7). Note: The UPC ratio cannot be predicted accurately from semiquantitative dipstick analysis and USG_{ref}.

$$\frac{\text{Protein excretion rate}}{\text{Creatinine excretion rate}} = \frac{\frac{[\text{Prot}]_u}{[\text{Prot}]_s} \times \text{volume}_u \div \text{time} \div \text{body weight}}{\frac{[\text{Crt}]_u}{[\text{Crt}]_s} \times \text{volume}_u \div \text{time} \div \text{body weight}} \quad (8.7)$$

For a randomly collected urine sample, some factors in the numerator and denominator are either the same or remain relatively constant:

- Urine volume, time over which the urine formed, and body weight values are the same.
- Serum [Prot] and [Crt] were probably constant during the time the urine formed.

Therefore, the relative rate of protein excretion compared to Crt excretion can be estimated as follows:

$$\frac{\text{Protein excretion rate}}{\text{Creatinine excretion rate}} = \frac{\frac{[\text{Prot}]_u}{\text{constant}}}{\frac{[\text{Crt}]_u}{\text{constant}}} \propto \frac{[\text{Prot}]_u}{[\text{Crt}]_u} = \text{UPC ratio}$$

B. Analytical concepts

1. Unless there is severe proteinuria, urine protein concentrations are below the analytical range of serum protein assays. Thus, different assays for low protein concentrations (often collectively called *microprotein assays*) are needed to measure [protein] in most urine samples. As noted above, there can be poor agreement among urine protein assays because of the different abilities of the assays to react with certain proteins and the different proteins that are used as assay calibrators/standards. Also, there can be differences among laboratories that are using the same type of urine protein assay. When urine samples from 105 dogs were analyzed with the pyrogallol red assay in three different laboratories, the mean bias of Lab 1 vs Lab 2 results was minimal but there were differences of more than 50 mg/dL for some samples. The mean biases for Lab 3's results versus Lab 1 or Lab 2 were about 30 mg/dL with a proportional positive bias leading to differences greater than 100 mg/dL.[230] This is enough to alter diagnostic interpretation of the UPC ratio in some patients.
2. Most analytical methods designed for measuring serum [Crt] are used for measuring urine [Crt], but the assays are rarely validated for urine samples.[231] For urine samples, the urine must be diluted to produce a [Crt] that is within the analytical range of the assay, and varying the dilution ratio can cause inaccuracies.[216] Some benchtop analyzers have been shown to under-measure urine [Crt] by approximately 25 % and thus produce higher UPC ratios.[216] As noted in the serum Crt section and shown in Table 8.5, clinical Crt assays can measure different concentrations when the same sample is analyzed.
3. Considering the analytical differences and inaccuracies of the urine protein and Crt assays,[216] calculated UPC ratios will vary among laboratories, as should RIs and medical decision limits. When UPC ratios were determined on 105 canine urine samples by three laboratories (each using the pyrogallol red assay), the average differences were near 0.0, but several differences were greater than 1.0 (e.g., ratio of 4.0 by one laboratory and a ratio of 1.2 by another laboratory).[230]

4. A reagent pad method of estimating UPC ratio (Siemens Multistix Pro) provides semiquantitation of Crt (10, 50, 100, 200, and 300 mg/dL) and protein (negative, 15, 30, 100, 300, or 2000 mg/dL). A low-protein pad is designed to detect albumin concentrations of at least 8–15 mg/dL via binding to a sulfonephthalein dye (reported as negative, 15 mg/dL, or 30 mg/dL), while a high-protein pad uses tetrabromophenol blue and the protein-error-of-indicator principle to detect albumin and other proteins when present at concentrations of at least 30–65 mg/dL. These UPC estimates appeared to be unreliable in cats whether calculated manually or based on a Clinitek 50 result.[232] With canine samples, UPC ratios calculated from the protein and Crt dipstick results yielded many false-negative values for a quantitative UPC ratio > 0.5, while the Clinitek 50 results were often falsely positive.

C. Correlation of 24-h protein excretion and UPC ratio
   1. Dogs: When dogs with potential nonrenal proteinuria were excluded, there was good correlation between the UPC ratio and the quantity of urinary protein excreted per day (either mg/d or mg/kg/d).[233–235] Results of three studies are compared in Table 8.18. Note that the highest ratios for healthy dogs in the three studies were 0.54, 0.17, and 0.38 with two different protein assays, and the 0.54 and 0.38 values were from the same type of assay. Although differences in reference sample groups may have contributed to differences in the ratios, method differences also likely affected the results.[234] UPC ratios are not influenced by collection period (day or night) or gender of dogs.[237]
   2. Horses: When using a Coomassie brilliant blue assay and 24-hr urine collection, the UPC ratios in 11 horses and 6 ponies were < 0.5 in pooled 24-hr collected urine and < 0.2 in pooled 4-hr collected urine. The ratios correlated well with the quantity of urinary protein excreted per day.[238]

D. Substaging of proteinuria in dogs and cats by IRIS
   1. In addition to creating stages for chronic renal disease, IRIS has proposed guidelines for proteinuria (www.iris-kidney.com). Different decision thresholds may be appropriate for different protein and Crt methods.
   2. As noted for the staging of chronic renal disease using serum Crt concentrations, these guidelines should not be used as firmly established decision limits because of the analytical variation that is present among urine protein and Crt assays.

Table 8.18. UPC ratios and 24-h urinary protein excretion studies in dogs

	Group	White[233a,b]	Grauer[235b,c]	Center[236a,b]
UPC ratio	Healthy	0.08–0.54 (8)[d]	0.02–0.17 (16)	0.01–0.38 (19)
	Proteinuric[e]	1.09–8.63 (10)	0.48–15.1 (14)	0.47–46.65 (38)
24-h urinary protein excretion (mg/kg)	Healthy	1.9–11.7 (8)	0.6–5.1 (16)	0.2–7.7 (19)
	Proteinuric	32.2–271.1 (10)	12.2–287.5 (14)	7.5–533.7 (38)[f]

[a] Protein method: trichloroacetic acid protein method
[b] Crt method: alkaline picric acid
[c] Protein method: Coomassie brilliant blue
[d] The number in parenthesis is the number of dogs in a study group.
[e] Dogs with or suspected of having either prerenal or postrenal proteinuria were excluded from the studies.
[f] The dog with a protein loss of 7.5 mg/kg was included in the proteinuric group because it had glomerulonephritis and its UPC ratio was considered increased (0.47).

E. Diagnostic significance of increased UPC ratio
1. The UPC ratio should be increased in any animal with proteinuria, including overflow, glomerular, tubular, inflammatory, and hemorrhagic proteinuria. Other clinical information is used to differentiate causes of the proteinuria (see Chemical Examination of Urine, Section III.C).
2. Glomerular proteinuria tends to be more severe and cause hypoalbuminemia or hypoproteinemia. However, earlier stages of glomerular damage may cause only mild proteinuria and no hypoalbuminemia (e.g., UPC ratio < 1.0). In the absence of hyperproteinemia and increased cell concentrations in urine, large increases in the UPC ratio (e.g., > 5.0) support glomerular proteinuria.[239] UPC ratios can be > 20–30 in dogs with glomerulosclerosis, immune complex–mediated glomerulonephritis, or amyloidosis.
3. Blood contamination of urine increases the UPC ratio, the degree of change depending on the amount of blood and the blood's Hct and [protein]. Sufficient blood to impart an orange color to urine can raise the UPC ratio from a nonproteinuric to a proteinuric interpretation, and addition of less blood such that urine remains yellow can change an interpretation from negative to borderline or borderline to increased.[240,241] With 5 % blood contamination, some canine samples had a change in UPC ratio from < 0.2 to > 2.0.

VI. Urine albumin assays
A. General concepts
1. Mammalian glomeruli are poorly permeable to albumin because it has a strong negative charge and the albumin molecular diameter (36 Å) is close to the diameter of a glomerular pore (42 Å).[128] However, the GFB does allow a small amount of albumin and slightly smaller proteins to pass into the filtrate; most of these proteins are resorbed by the PCTs, some by the LoH and distal nephron.[242] If there is minor damage to the barrier (a reduced negative charge or damaged podocytes), then more albumin enters the tubular fluid. Incomplete resorption of the albumin causes albuminuria. With increasing damage, larger proteins (various globulins) also pass into the tubular fluid.
2. In urine samples from healthy people, renal excretion of albumin results in a urine [albumin] that is typically < 3 mg/dL, so < 30 mg of albumin are typically excreted per day.[243] Consequently, human microalbuminuria has been defined as a urinary [albumin] of 2–20 mg/dL.[244] Using regular UA reagent strips, such albumin concentrations might create trace protein reactions.
3. For many years, while the protein reaction of a typical UA was expected to be negative in the urine of healthy cats, horses, and cattle, a trace or 1+ reaction in concentrated canine urine was accepted as a species variation and not evidence of a pathologic state.[140] Moreover, bands corresponding to albumin are often present in electrophoresis gels of urine from clinically healthy dogs.[142–144, 160] However, more recently, some considered the mild proteinuria in clinically healthy dogs evidence of subclinical glomerular disease.[245] Consequently, assays were developed to measure low urine albumin concentrations, i.e., so-called microalbuminuria.
4. The working definition of *microalbuminuria* in dogs and cats is urine albumin concentrations from 1 mg/dL to 30 mg/dL in urine adjusted to a USG_{ref} of 1.010.[245,246] Concentrations > 30 mg/dL are referred to as *albuminuria* or *overt albuminuria*. It is important to remember that a urine [albumin] of 30 mg/dL in urine with a USG_{ref} of 1.010 would have an [albumin] of 60 mg/dL if the USG_{ref} were 1.020,

or 120 mg/dL if the USG_{ref} were 1.040 (the same amounts of albumin loss but with less H_2O loss).

5. Some authors state that 30 mg/dL is the decision threshold for microalbuminuria because common UA methods do not detect protein concentrations < 30 mg/dL. However, the Chemstrip package insert states that, in 90 % of urine samples tested, protein concentrations ≥ 6 mg/dL produced a color change. For the Multistix system, a trace reaction corresponds with an estimated [protein] of 10 mg/dL. When turbidity standard solutions are used with the SSA test, increased turbidity can be detected when the [protein] is 5 mg/dL.
6. It is important to remember that microalbuminuria is not unique to glomerular disease and occurs in some clinically healthy dogs.[247] It may be caused by any of the disorders and conditions that cause proteinuria (glomerular, tubular, hemorrhagic, or inflammatory). Thus, the presence of microalbuminuria should be interpreted in the context of other clinical information. Clinical information (history, physical examination findings, UA, and other routine laboratory results) typically enables one to recognize an overflow, hemorrhagic, or inflammatory proteinuria. It is more difficult to differentiate glomerular from tubular proteinuria.[167]
7. The effects of urinary tract inflammation or hemorrhage on the urine [albumin] were explored by using a capture ELISA for canine [albumin] that the authors acknowledged was not fully validated.[246] In 70 dogs with pyuria, 23 had increased urine [albumin] (≥ 1 mg/dL). Of the 70 dogs with pyuria and hematuria, 25 had increased urine [albumin].
8. In contrast to findings in people, exercise did not increase the urinary excretion of albumin in 26 dogs.[247]

B. Analytical concepts
   1. Quantitative immunologic assay
      a. The immunologic microalbumin assays are species specific because of the species differences in albumin molecules. ELISAs and nephelometric assays have been developed for canine and feline urine.[244]
      b. The standard solutions for the assays are canine or feline albumin. For one ELISA, standard solutions were diluted to create an analytical range of 1.9–30 ng/mL (0.19–3.00 μg/dL).[244] Urine samples were diluted (up to 1:3200) to obtain urine albumin concentrations within the analytical range of the assay; such marked dilutions can be a source of analytical error.[248]
      c. One method of creating a quantitative, species-specific assay is to modify a human microalbumin assay by using canine or feline albumin as the calibrator (standard). When done with one assay, there was sufficient cross-reactivity of the goat anti-(human albumin) antibody to generate a standard curve for canine urine.[249] When human albumin was used in the calibrator solution, the measured albumin concentrations were about half of those obtained when canine albumin was used as the calibrator. However, reliable use of an anti-(human albumin) antibody for assessing low concentrations of albumin in other species requires confirmation that the antibody does not bind to other proteins, and this was not shown. Also, an assay's lower detection limit for albumin and its performance at low albumin concentrations must be validated.
      d. As for urine protein assays and especially for immunologic assays, a microalbumin assay may not detect albumin fragments that have undergone degradation.[250]

2. Albumin-to-Crt ratio
   a. Siemens markets a reagent pad method of estimating an albumin-to-Crt ratio (Clinitek Microalbumin). The albumin reaction is a dye-binding method with color changes representing 0, 1, 3, 8, 15, and 50 mg/dL. The Crt reaction is a peroxidase-like assay with color changes corresponding to 30, 100, 200, and 300 mg/dL. When the color changes are read by reflectance photometry in a Siemens instrument (Clinitek), an estimated albumin-to-Crt ratio is calculated. The reported units of the ratios vary: 300 mg/g = 300 μg/mg = 0.3 mg/mg = 0.3 (unitless).
   b. The albumin assay is not specific for albumin. Many other proteins can react with the dye, including immunoglobulin light chains, whole immunoglobulins, β_2-microglobulin, uromodulin, hemoglobin, and myoglobin.[251] However, other than hemoglobin and myoglobin, most of these proteins are usually not in urine at sufficient concentrations to produce a false-positive albumin reaction.
   c. In one comparison study using canine urine samples, the authors concluded that the available dipstick method (Clinitek Microalbumin) was not reliable for detection of microalbuminuria. The results of a dipstick method were compared to the results of a canine albumin-capture ELISA; albumin concentrations were normalized to a specific gravity of 1.010.[244] The Siemens albumin-to-Crt ratio method correctly classified 48 % of the microalbuminuric samples (i.e., those with albumin concentrations of 1–30 mg/dL) as microalbuminemic. Some samples were hematuric (RBCs > 5/hpf, > 1+ heme reaction), and hemoglobin can bind to the reagent dye.[251] Thus, some discrepancies may have been due to proteins other than albumin binding to the reagent in the dipstick.
3. Comparison to other urine protein assays
   a. [Protein] estimated by routine protein assays of urinalyses (i.e., the reagent pad method and SSA turbidity) represents the sum of albumin and globulin concentrations. Both methods detect albumin better than most globulins, but globulins contribute to the reactions. The detection limits of reagent pad methods vary (Table 8.12).
   b. The SSA assay is also called the *bumintest* because it detects albumin better than globulins. When a sample's result is compared to standard solutions, turbidity can be detected at an [albumin] of 5 mg/dL. If very mild proteinuria is caused by glomerular disease, albumin is the dominant protein in the urine.
   c. The results of these routine protein assays are typically not normalized to a specific gravity of 1.010. However, their results should be interpreted with knowledge of the sample's USG_{ref} so that an estimated adjustment can be made. In a concentrated urine sample in which microalbuminuria is detected after normalization to 1.010, it would not be unusual for common protein dipstick reactions to produce a trace or 1+ reaction or for the SSA assay to have 1+ turbidity.

VII. Urinary enzyme activities to assess renal tubular damage
   A. Theory
      1. Damage to PCT cells releases cellular enzymes and thus increases the amount of those enzymes in an animal's urine. AAP, GGT, and ALP are present in the cells' brush borders, whereas N-acetyl-β-D-glucosamidase (NAG) and β-glucuronidase are lysosomal enzymes,[252] and lactate dehydrogenase is a cytoplasmic enzyme. The enzymes are considered markers of proximal tubular cell damage – the primary site of damage when there is acute toxic nephrosis. However, increased renal excretion of

lysosomal enzymes might reflect increased lysosomal turnover via endocytosis and exocytosis in addition to tubular damage.[253]

2. Because these enzymes are proteins, rather than reflecting renal tubular damage, these enzymes could be in the urine if they passed through the GFB when there is glomerular proteinuria. Thus, assessment of urinary enzyme excretion should be made with consideration of the presence or absence of glomerular proteinuria. If there is glomerular proteinuria and the plasma enzyme activity (e.g., ALP, GGT) is sufficiently increased, then it may be difficult to determine if the enzyme activity is of renal or plasmatic origin. In addition, ALP activity may originate from prostatic secretions of male dogs.
3. As with other chemical analytes in urine, direct interpretation of urine enzyme activity concentration (U/L) is difficult because of variations in renal excretion of H_2O. Thus, enzyme data are commonly related to urinary Crt excretion (e.g., U of enzyme per g of Crt), but other methods have been used.[252] When comparing data, one should be aware of variations in assays and units (Crt in mg/dL versus μmol/L). An increased enzyme to Crt ratio typically reflects tubular damage; however, acute nephron damage could decrease Crt entry into tubular fluid and concurrently increase entry of enzymes into the tubular fluid. The combination would increase the enzyme to Crt ratio more than if there were only tubular damage.

B. Analytical concepts
1. The commercial assays that are designed for measuring enzyme activity in plasma or serum may need modification for use with urine samples.
2. Similar to human urine, canine urine may contain inhibitors of urinary enzymes. One study demonstrated that gel filtration was needed to remove the AAP inhibitor in all canine urine samples and to remove the NAG inhibitor in most canine urine samples.[254]
3. It has been shown that urine pH and urine pigments can interfere with the measurement of NAG activity. In urine of greater alkalinity, NAG activity decreased because of enzyme instability, whereas urine pigments could falsely increase the measured activity.[255] In another study, it was shown that the decrease in total NAG activity in alkaline urine was mostly due to the instability of NAG isoenzyme A.[256]
4. Canine urine GGT activity decreased when the dipstick pH of samples was outside the 6.5–8 interval.[257] GGT activity in seminal fluid may contribute to urine GGT activity in male dogs.
5. Storage of urine
   a. AAP and NAG activities were considered stable for 24 h at room temperature and 4 °C. AAP activity deteriorated greatly after 1 mo and slightly after 4 d at –20 °C, but was stable at –70 °C for 1 mo; NAG remained stable at –20 °C and –70 °C for 1 mo.[258]
   b. Lactate dehydrogenase, GGT, and ALP activities were considered stable in human urine for 24 h at room temperature and 4 °C but were not stable at –20 °C or –70 °C (likely enzyme damage during freezing and thawing).[257,259] Canine urine GGT activity decreased quickly and markedly with freezing at –20 °C, but activity was stable for 3 d at 4 °C and 20 °C.[257]

C. Increased urinary enzyme activity can help detect ongoing or newly developing renal disease in at-risk azotemic or nonazotemic groups such as critically ill patients under intensive care or those receiving aminoglycoside antibiotics.

1. In 15 horses (six healthy, nine with pleuropneumonia) given recommended doses of gentamicin for 10 d, all treated horses had increased urine GGT to Crt ratios (about double pretreatment ratios) by day 2 or 3 after initiation of treatment, and the increases persisted up to 10 d after treatment.[260]
2. In six healthy horses given four therapeutic doses of imidocarb dipropionate at 72-hr intervals, urine GGT to Crt ratios were increased by day 5 and remained increased until 10 d after the last dose.[261]
3. In 10 azotemic dogs with chronic renal disease, the urine NAG to Crt ratios were greater than those found in clinically healthy dogs (samples frozen at –80 °C prior to analysis).[262] The presence or absence of alkalinuria was not reported (NAG is less stable in alkaline urine), and it was not stated if gel filtration was completed before analysis.
4. In 15 dogs with renal lesions associated with leishmaniasis, the urine NAG to Crt, GGT to Crt, and β-glucuronidase to Crt ratios were greater than those of eight healthy dogs.[263] As above, the presence or absence of alkalinuria was not reported; gel filtration was completed prior to storage at –20 °C. Additional studies of tubular enzymuria have supported the presence of transient or persistent tubular damage in proteinuric dogs with leishmaniasis.

VIII. Other biomarkers of renal injury

A. Although other plasma or urinary biomarkers of renal injury have been identified and measured, there is generally limited knowledge of their clinical value in veterinary medicine. These biomarkers offer the potential for better differentiation of renal disorders, earlier detection of acute renal injury, and monitoring the progression of chronic renal disease. Clinical use requires appropriate analytical validation of assays followed by demonstration of useful diagnostic performance that exceeds that of current biomarkers or is similar but with other practical advantages. Information pertaining to many of the potential biomarkers evaluated in dogs, cats, and horses has been reviewed.[264,265]

B. Biomarkers for glomerular damage (analyte concentrations are typically normalized to [Crt] as ratios)
   1. Leaky GFB: increased urine concentrations of C-reactive protein, IgG, or transferrin
   2. Podocyte damage: increased urine [podocin]
   3. Decreased GFR: increased serum or plasma [cystatin C]

C. Biomarkers for tubular damage (analyte concentrations often normalized to [Crt] as ratios)
   1. Impaired tubular resorption: increased urine concentrations of cystatin C, β_2 microglobulin, neutrophil gelatinase-associated lipocalin, or retinol binding protein
   2. Release from damaged tubular epithelial cells (± upregulated production by tubular cells): increased urine concentrations of clusterin, cystatin B, heat shock protein 72, kidney injury molecule-1, liver-type fatty acid–binding protein, or neutrophil gelatinase-associated lipocalin
   3. Decreased production by tubular epithelial cells: decreased urine concentration of uromodulin

D. Other miscellaneous biomarkers
   1. Increased urine concentrations of interleukins (6, 8, 18), F_2-isoprostanes, monocyte chemoattractant protein 1, thromboxane B2, glutathione S-transferase, hyaluronic acid, osteopontin, ferritin, matrix metalloproteinases (5, 9), and acid phosphatase
   2. Decreased serum concentration of uromodulin

IX. Fractional excretion (FE) ratios or percentages

A. Theory

1. The FE of substance X reflects the relative rate of urinary excretion of substance X compared to Crt. If substance X passes freely through the GFB and is neither secreted nor resorbed (as is nearly true for Crt), the FE would be 1.0. If some of substance X were resorbed after freely entering the filtrate, the FE would be < 1.0. Therefore, for solutes that freely pass the GFB (e.g., electrolytes), FE is the fraction of the solute entering the filtrate or tubular fluid that is ultimately excreted. Substance X can be an electrolyte, an enzyme, or another solute. For the derivation of the FE formula (Eq. 8.8), substance X is Na^+.

$$\frac{\text{Urinary } Na^+ \text{ excretion rate}}{\text{Urinary creatinine excretion rate}} = \frac{\frac{[Na^+]_u}{[Na^+]_s} \times \text{volume}_u \div \text{time} \div \text{body weight}}{\frac{[Crt]_u}{[Crt]_s} \times \text{volume}_u \div \text{time} \div \text{body weight}} \quad (\mathbf{8.8})$$

For a randomly collected urine sample:

- Urine volume, time over which urine formed, and body weight values are the same in the numerator and denominator and thus cancel out.
- Serum $[Na^+]$ and [Crt] are likely nearly constant as urine forms, but they can vary.
- Urine $[Na^+]$ and [Crt] are expected to vary because of multiple factors.

Therefore, the relative rates of Na^+ and Crt urinary excretion can be expressed as:

$$\text{Fractional excretion of } Na^+ (\text{FE } Na^+) = \frac{Na^+ \text{ excretion}}{\text{Creatinine excretion}} = \frac{\frac{[Na^+]_u}{[Na^+]_s}}{\frac{[Crt]_u}{[Crt]_s}} = \frac{[Na^+]_u}{[Na^+]_s} \times \frac{[Crt]_s}{[Crt]_u}$$

$$\text{Expressed as a percentage, percent FE } Na^+ = \frac{[Na^+]_u}{[Na^+]_s} \times \frac{[Crt]_s}{[Crt]_u} \times 100$$

2. A major advantage of FE over 24-h excretion is that the assessment can be done on a single urine sample. However, the urine and serum (or plasma) samples for the assessment should be collected near the same time. A FE value is typically a better assessment of renal function than a simple ratio of urine analyte concentration to plasma analyte concentration because FE accounts for variations in renal excretion caused by altered GFR.

B. Interpretive concepts

1. A FE ratio provides the relative rate of excretion of an analyte compared to Crt. For a FE ratio to reflect the 24-h urinary excretion of an analyte accurately, Crt excretion needs to be within the RI and relatively constant.
2. An increased FE ratio may reflect increased urinary excretion of an analyte. This can occur for the following reasons.
   a. Plasma analyte concentrations are increased (resulting in increased filtered load), and the kidneys are attempting to excrete the excess. This may occur with increased dietary intake of the analyte.
   b. Increased tubular secretion of the analyte
   c. Decreased tubular resorption of the analyte

3. A decreased FE ratio of an analyte may result from the opposite processes:
   a. Plasma analyte concentrations are decreased (resulting in decreased filtered load), and the kidneys are attempting to conserve the analyte. This may occur with decreased dietary intake of the analyte.
   b. Decreased tubular secretion of the analyte
   c. Increased tubular resorption of the analyte
4. An increased FE ratio may also occur with decreased Crt excretion.
   a. An increased FE of K^+ would occur if the renal excretion of K^+ is maintained through secretion of K^+ but Crt excretion is decreased because of decreased GFR.
   b. FE of PO_4 may be increased because of decreased GFR (thus less filtration of Crt and PO_4) or when tubular resorption of filtered PO_4 is decreased because of increased PTH activity.

C. The assays used to measure analyte concentrations in serum or plasma may not provide accurate results for urine samples.
1. Urine can contain an inhibitor that interferes with the ion-selective electrode assay for $[K^+]$.[220]
2. Urine may need to be acidified to promote dissociation of ion complexes, e.g., Ca^{2+} dissociation from PO_4 or other anions that are within urine crystals.
3. Some assay systems require the protein matrix of serum or plasma; the nearly protein-free fluid (urine) may not be acceptable.

D. FE studies are not commonly used in veterinary medicine because information obtained may not be required for diagnosis and case management. Also, appropriate RIs are difficult to obtain for each species, especially when one considers that separate RIs may be needed for different assay systems and different diets. The major application of FE methods is for nutritional studies and renal research, for which conditions affecting FE can be well controlled.[231]

X. Bladder tumor antigen test

A. A latex agglutination test was developed for the detection of a glycoprotein complex that is common in the urine of people with transitional cell (urothelial cell) carcinoma. The human assay was used in a study involving 20 dogs with transitional cell carcinomas. Eighteen of the 20 dogs had positive results for the bladder tumor antigen. However, diagnostic specificity suffered from false-positive results in dogs with marked glucosuria, marked proteinuria, and either hematuria or pyuria.[266]

B. A similar veterinary bladder tumor antigen (V-BTA) assay was developed in which the latex particles were coated with human IgG reactive with the target antigen.
1. As in the previous study, positive results were found for dogs with malignant genitourinary tract neoplasms, including transitional cell carcinoma and prostatic adenocarcinomas. However, positive results were found in 11 of 17 dogs that had nonmalignant urinary tract disease.[267]
2. In another study, positive results were found for most dogs with malignant genitourinary tract neoplasms including transitional cell carcinoma and prostatic neoplasms. However, positive results were found in dogs that had nonmalignant urinary tract disease and in unhealthy dogs without urinary tract disease.[268]

H_2O DEPRIVATION AND ANTIDIURETIC HORMONE (ADH) RESPONSE TESTS IN ANIMALS WITH POLYURIA AND POLYDIPSIA (PU/PD)

Renal concentrating ability is assessed in a routine UA by determining the USG_{ref}. Typically, the USG_{ref} along with other case information (historical and physical findings and other laboratory data) enables veterinarians to identify the probable causes of a PU/PD disorder (Tables 8.10 and 8.11).

When a more critical assessment of renal concentrating ability is needed, and especially if central diabetes insipidus is suspected, either H_2O deprivation or ADH response tests may be considered. Because interpretation guidelines may differ when specific aspects of these tests vary, this section is limited to the major concepts (reference articles or texts should be consulted for specific interpretation guidelines).

I. Abrupt H_2O deprivation test[64]
   A. General concepts
      1. The basic purpose is to assess the ability of the kidneys to concentrate the filtrate by inducing an abrupt stimulus (hypovolemia or hyperosmolality) for renal H_2O retention. The test may be used to evaluate undiagnosed PU/PD patients that consistently have urine with $USG_{ref} < 1.020$.
      2. The test is contraindicated in azotemic animals (or those known to have decreased GFR) or in dehydrated patients (already challenged to concentrate). The procedure can be dangerous in severe PU states because the animal can quickly become severely hypovolemic.
   B. Basics of the procedure
      1. Baseline information is collected and may include body weight, USG_{ref}, urine and serum osmolality, and serum $[Na^+]$.
      2. Access to H_2O is abruptly removed, the animal is monitored, and the findings are compared with baseline information until they indicate one of the following:
         a. The animal has become dangerously dehydrated.
         b. The kidneys can concentrate urine.
         c. The animal was adequately challenged, and its kidneys did not adequately concentrate urine.
   C. Basic interpretations
      1. If the animal demonstrates the ability to concentrate urine (the USG_{ref} or urine osmolality criteria are exceeded), the PU/PD state is a primary PD disorder.
      2. If the animal does not demonstrate ability to concentrate urine, several possibilities exist.
         a. The procedure had to be halted before renal concentrating mechanisms produced concentrated urine.
         b. Secondary medullary hypotonicity may be present because of persistent polyuria from a variety of disorders.
         c. A disorder that causes nephrogenic diabetes insipidus is present.
         d. Central diabetes insipidus is present.

II. Gradual H_2O deprivation test[64]
   A. The purpose, indications, and contraindications are the same as for the abrupt H_2O deprivation test. The gradual H_2O deprivation test is indicated if medullary hypotonicity from prolonged PU (medullary washout) or other cause is expected or if there is failure to concentrate urine after abrupt H_2O deprivation. The gradual decrease in H_2O intake enables the kidneys to reestablish a medullary concentration gradient. It tends to be a less dangerous procedure in severely PU animals, but animals still must be monitored thoroughly for development of dehydration.
   B. The major difference from the abrupt deprivation procedure is that H_2O intake and urine output are first measured and recorded over two or more days, and then H_2O availability is decreased by 10 % each day. As for the abrupt test, the animal must be monitored for evidence of dangerous dehydration and ability to concentrate urine.

C. Advantages of the gradual H_2O deprivation are that it tends to be less dangerous and allows time for reestablishment of medullary hypertonicity. The major disadvantage is the time requirement; it may take over a week to complete the test.

III. ADH (vasopressin) response test[64]
A. The basic purposes are to assess the ability of the kidneys to respond to exogenous ADH and to confirm a complete or partial absence of ADH (diabetes insipidus) in a PU/PD patient. It may be more justified when H_2O deprivation does not lead to concentrated urine.
B. Basics of the procedure
1. H_2O access is ad libitum during the study.
2. After administration of aqueous vasopressin or synthetic deamino-d-arginine vasopressin (DDAVP), renal concentrating ability is assessed by measuring the USG_{ref} or urine osmolality.
C. Expected findings
1. Central diabetes insipidus: The kidneys did not concentrate urine during H_2O deprivation but did when stimulated by exogenous ADH.
2. Nephrogenic diabetes insipidus: The kidneys did not concentrate urine during H_2O deprivation or after stimulation by exogenous ADH. The same results may occur with medullary hypotonicity such as that caused by any primary PU or PD disorder.

IV. Modified H_2O deprivation test[269]
A. Combination of H_2O deprivation test and ADH response test
B. Procedure
1. The animal is abruptly deprived of H_2O and then the urine osmolality is measured hourly until the increase in urine osmolality between consecutive hourly samples is < 5 %. If there is a marked concentrating defect, the urine osmolality might not increase 5 % within 1 h.
2. ADH is administered and urine is collected 1 h after injection.
C. Expected results
1. Healthy dogs: urine osmolality after H_2O deprivation > 1000 mmol/kg, and a 0 % increase in urine osmolality after ADH administration
2. Partial central diabetes insipidus and partial renal diabetes insipidus (including dogs with hyperadrenocorticism): urine osmolality after H_2O deprivation < 600 mmol/kg, and a 20–50 % increase in urine osmolality after ADH administration
3. Central diabetes insipidus: urine osmolality after H_2O deprivation < 300 mmol/kg, and a > 100 % increase in urine osmolality after ADH administration
4. Primary polydipsia: The response should be similar to that of healthy dogs if the kidneys have medullary hypertonicity, but there may be a poor response if they do not.
5. Renal diabetes insipidus: The primary cause of renal diabetes insipidus is typically identified before a H_2O deprivation test is considered. Animals with renal diabetes insipidus would have H_2O deprivation test results similar to those for central diabetes insipidus but administered ADH may not override the underlying impaired tubular response to ADH.

UROLITH ANALYSIS

I. A *urolith* (*urinary calculus*) is a macroscopic solid concretion (stone) that forms within the urinary tract and typically is found in a renal pelvis, urinary bladder, urethra, or voided urine. Sand-like

(*sabulous*) urolithiasis occurs when many small concretions form. Learning the composition of a urolith provides evidence for the pathogenesis of its formation and aids in developing therapeutic plans to eliminate or to reduce chances for recurrence. Uroliths are found most commonly in dogs and are relatively uncommon in cats, horses, cattle, and llamas. Most urinary tract obstructions in cats are not caused by uroliths but by plugs consisting of phosphate crystals and mucoid material. Uroliths are not large urine crystals but consist of aggregated crystals and an organic matrix. Many crystals form into one or more typical three-dimensional structures that facilitate their identification. The chemical compositions of uroliths are listed in Table 8.19.

II. Information regarding the detection, analysis, treatment, and management of uroliths can be found in review articles[270–273] and medicine textbooks for individual species.

III. Analytical concepts

A. For many years, uroliths were analyzed chemically with a series of qualitative chemical reactions to detect the presence of certain cations and anions. These chemical methods are

Table 8.19. Inorganic composition of uroliths[a]

Group	Chemical name	Formula	Mineralogic name
Carbonate	Calcium carbonate	$CaCO_3$	Calcite, aragonite, vaterite
Cystine	Cystine	$SCH_2CH(NH_2)COOH$	Cystine
Oxalates	Calcium oxalate monohydrate	$Ca(C_2O_4)\cdot H_2O$	Whewellite
	Calcium oxalate dihydrate	$Ca(C_2O_4)\cdot 2H_2O$	Weddellite
Phosphates	Basic calcium phosphate	$Ca_5(PO_4)_3(OH)$	Apatite
	Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite
	Carbonate-apatite	$Ca_{10}(PO_4, CO_3OH)_6(OH)_2$	Carbonate-apatite
	Calcium hydrogen phosphate dihydrate	$CaHPO_4\cdot 2H_2O$	Brushite
	Tricalcium phosphate	$Ca_3(PO_4)_2$	Whitlockite
	Octacalcium phosphate	$CaH(PO_4)_3\cdot 2\cdot 5H_2O$	—
	Magnesium ammonium phosphate hexahydrate	$MgNH_4PO_4\cdot 6H_2O$	Struvite
	Magnesium hydrogen phosphate trihydrate	$MgHPO_4\cdot 3H_2O$	Newberyite
Silica	Silicone dioxide	SiO_2	Silica
Uric acids	Anhydrous uric acid	$C_5H_4N_4O_3$	Uricite
	Uric acid dihydrate	$C_5H_4N_4O_3\cdot 2H_2O$	—
Purines	Ammonium acid urate[b]	$C_5H_4N_4O_3NH_4$	—
	Sodium acid urate monohydrate	$C_5H_4N_4O_3Na\cdot H_2O$	—
	2,8-Dihydroxyadenine	$C_5H_5N_5O_2$	—

[a] A urolith is classified as a specific type if at least 70 % of its composition is composed of that mineral. If it does not meet that criterion, it is referred to as a mixed urolith.

[b] Also known as ammonium hydrogen urate or ammonium biurate

nearly obsolete because (i) they did not agree well with results of quantitative crystallographic methods, (ii) they did not provide a relative amount of each constituent, (iii) significant amounts of some ions were missed, (iv) they lacked methods for detecting silica and cystine, (v) false-positive results occurred for some compounds, and (vi) mixed uroliths could not be classified.[274]

B. The chemical methods have been replaced by physical analytical methods. These analyses are offered by a few specialized laboratories (e.g., the Minnesota Urolith Center, the Urolithiasis Laboratory at Baylor College of Medicine, the G.V. Ling Urinary Stone Analysis Laboratory at the University of California–Davis, and the Canadian Veterinary Urolith Centre at the University of Guelph).[275–277]
   1. Optical crystallography: Ground urolith material is examined with a polarizing microscope after the material is placed in a series of oils with different refractive indices. Each crystal is identified when its refractive index is matched with one of the oils (i.e., it disappears). Different layers of the urolith are analyzed to determine the composition of each layer.
   2. X-ray diffraction: Ground urolith material is bombarded with X-rays. As the X-rays hit a crystal, they are diffracted (scattered) in a unique way for each crystal. The diffractions are recorded to determine the chemical composition of the urolith.
   3. Infrared spectroscopy: Infrared waves are directed at the sample and waves that are absorbed or transmitted by the sample are detected. Each molecular structure has a unique spectral pattern that identifies the composition of the urolith.
   4. Other less common methods include electron microprobe analysis and scanning electron microscopy.

IV. Pathogenesis of urolith formation: Urolith formation is a complex process that involves many factors that promote and others that inhibit formation. The major steps of urolith formation are as follows:[278]
   A. The cations and anions of the urolith are present at supersaturated concentrations in urine.
   B. The cations and anions combine into a unique crystalline structure. Factors such as pH, temperature, and flow rate influence crystallization. This occurs most easily when debris, casts, or other urine particles are present to provide an initiating surface, a process called *heterogeneous nucleation*. Crystals usually do not grow fast enough to become lodged in the urinary tract; instead, they are flushed out via the urine and may be seen in urine sediment.
   C. Crystals can combine into aggregates relatively quickly. If small aggregates are not passed, then they provide a surface for more crystallization and urolith formation.
   D. The formation of crystals and aggregates is naturally inhibited by a variety of substances. For example, citrate binds Ca^{2+} and thus limits its availability for crystal formation. Uromodulin is produced by the renal tubular cells and is a natural inhibitor of aggregation.
   E. Variable amounts of protein-rich organic matrix are incorporated into uroliths.

V. Predisposing or contributing factors for urolith formation: Factors that contribute to crystalluria typically also contribute to urolith formation. In addition, increased amounts of noncrystalline matrix and lower amounts of inhibitors promote aggregation.
   A. Struvite uroliths are associated with urinary tract infections, especially by urease-producing bacteria, which degrade urea to NH_4^+, thereby increasing the urine's alkalinity through the incorporation of free H^+. They are relatively common in dogs and cats.
   B. Calcium phosphate uroliths are associated with urinary tract infections (see struvite) and hypercalciuria caused by hypercalcemia.

C. Calcium oxalate uroliths occur with increased urinary excretion of Ca^{2+} associated with hypercalcemia or increased intake of Ca^{2+} or vitamin D. They also occur with increased urinary excretion of oxalate caused by ingestion of material with high oxalate content (chocolate, nuts, spinach, sweet potatoes, wheat germ, and oxalate-containing plants such as *Halogeton glomeratus*). They are relatively common in dogs and cats. Several small dog breeds are predisposed, including French bulldogs and English bulldogs.
D. Urate uroliths are found disproportionately in Dalmatians and English or American bulldogs because of the high renal excretion of urate in these breeds. They are also found in dogs with portosystemic shunts, hepatic microvascular dysplasia, or hepatic insufficiency. An increased incidence has been reported in Egyptian Mau cats.[279]
E. Cystine uroliths are found in dogs that have defective renal tubular resorption of amino acids including cysteine, thus leading to cystinuria. Affected breeds include English bulldogs, Newfoundlands, dachshunds, mastiffs, bullmastiffs, Australian cattle dogs, Scottish deerhounds, miniature pinschers, Labrador retrievers, Staffordshire bull terriers, and Yorkshire terriers. At least one type of cystinuria appears to be sex-linked, occurring in males, particularly intact males.
F. Xanthine uroliths are found in dogs treated with allopurinol (a xanthine oxidase inhibitor) for leishmaniasis or to control urate urolithiasis.[280] They also occur in rare dogs with hereditary xanthinuria (includes Cavalier King Charles spaniel, dachshund, Manchester terrier, English cocker spaniel, and Chihuahua breeds). Xanthinuria and xanthine urolithiasis also occur in cats.
G. Silica urolith formation in dogs has been associated with ingestion of diets containing corn gluten feed or soybean hulls, and with drinking H_2O with relatively high silica concentrations.[281] Silica uroliths may form in cattle grazing silica-rich soils, and silica urolithiasis has been reported in a llama.[282]
H. 2,8-Dihydroxyadenine uroliths have been reported in dogs[283] and a cow.[284] These rare purine uroliths may be mistaken for urate uroliths (also purine), but they are not associated with hepatic dysfunction.
I. Calcium carbonate uroliths occur in horses, but uncommonly.[273]

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 9

MONOVALENT ELECTROLYTES AND OSMOLALITY

Steven L. Stockham and Michael A. Scott

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Table 9.1. Abbreviations and symbols in this chapter	
[x]	x concentration (x = analyte)
AcAc	Acetoacetate
AcCoA	Acetyl-coenzyme A
ADH	Antidiuretic hormone
AG	Anion gap
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BHB	β-Hydroxybutyrate
DCT	Distal convoluted tubule
ECF	Extracellular fluid
EDTA	Ethylenediaminetetraacetic acid
fCa^{2+}	Free ionized calcium
fMg^{2+}	Free ionized magnesium
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
ICF	Intracellular fluid
IV	Intravenous or intravenously
LD	L-Lactate dehydrogenase
LoH	Loop of Henle
mA^-	Measured anion charge
mC^+	Measured cation charge
M_r	Relative molecular mass
$Na^+:K^+$	Sodium to potassium
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NH_4^+	Ammonium
Osm_c	Calculated osmolarity
Osm_m	Measured osmolality
Osmole gap	Difference between measured osmolality and calculated osmolarity
$pCO_2(a)$	Partial pressure of carbon dioxide in arterial blood
pH	$-\log [H^+]$
Pi	Inorganic phosphorus
pK_a	$-\log (K_a)$ where K_a is the ionization constant for a partially ionized acid
PO_4	Phosphate, all forms
PCT	Proximal convoluted tubule
RAAS	Renin–angiotensin–aldosterone system
RBC	Red blood cell (erythrocyte)
RI	Reference interval
SI	Système international d'unités (International System of Units)
SID	Strong-ion difference
SO_4	Sulfate, all forms

Table 9.1. Abbreviations and symbols in this chapter (Continued)	
tA^-	Total anionic charge
tbH_2O	Total body water content
tbK^+	Total body potassium content
$tbNa^+$	Total body sodium content
tC^+	Total cationic charge
tCO_2	Total carbon dioxide content
uA^-	Unmeasured anion charge
uC^+	Unmeasured cation charge
WRI	Within reference interval

BASIC CONCEPTS FOR THE INTERPRETATION OF ELECTROLYTE CONCENTRATIONS

I. Electrolyte concentrations in plasma are the net result of intake, excretion, and shifts between ICF and ECF. Shifts may occur in vivo or in vitro. The basic concepts for electrolyte and H_2O movements in and out of plasma are shown in Figure 9.1. Electrolyte concentrations in plasma essentially represent the electrolyte concentrations in all ECF, as capillaries are permeable to H_2O and unbound electrolytes.
   A. Na^+, K^+, and Cl^- typically enter the body via oral intake of food or fluids. HCO_3^- typically is generated by carbonic anhydrase reactions in lungs, gastric mucosa, kidneys, and RBCs.
   B. Outside the vascular system, metabolic processes govern the movement of electrolytes into and out of cells.
      1. In health, ECF is relatively rich in Na^+ and Cl^- but poor in K^+, whereas ICF is relatively rich in K^+ but poor in Na^+ and Cl^- (except in RBCs of some species and breeds).
      2. Electrolyte shifting from or to the ECF alters plasma concentrations of those electrolytes.
   C. Cells within blood may alter plasma electrolyte concentrations if they release electrolytes.
      1. In vitro lysis of K^+-rich RBCs results in falsely elevated plasma $[K^+]$. However, RBCs are K^+ rich in only some species and breeds (see Potassium Concentration, Section III.B.1.g).
      2. The $[K^+]$ is slightly greater in serum than in plasma because platelets release K^+ during clotting. The magnitude of the increase can be clinically significant when there is a thrombocytosis.
   D. Electrolytes and H_2O are excreted or lost from the ECF via kidneys, alimentary tract, skin, and airways. Drinking H_2O after the loss of ECF dilutes the remaining ECF.
   E. HCO_3^- concentrations reflect concentrations of other electrolytes and changes in acid-base balance.

II. Abnormal electrolyte concentrations in plasma result from one or more of these basic processes involving electrolytes or H_2O:
   A. Decreased or increased intake
   B. Shifts to and from ICF
   C. Increased renal retention
   D. Increased loss via the kidneys, alimentary tract, skin, or airways (HCO_3^- indirectly)
   E. Increased production of HCO_3^- by gastric mucosal or renal epithelial cells
   F. Shifting to or from a cavitary effusion (e.g., uroperitoneum)

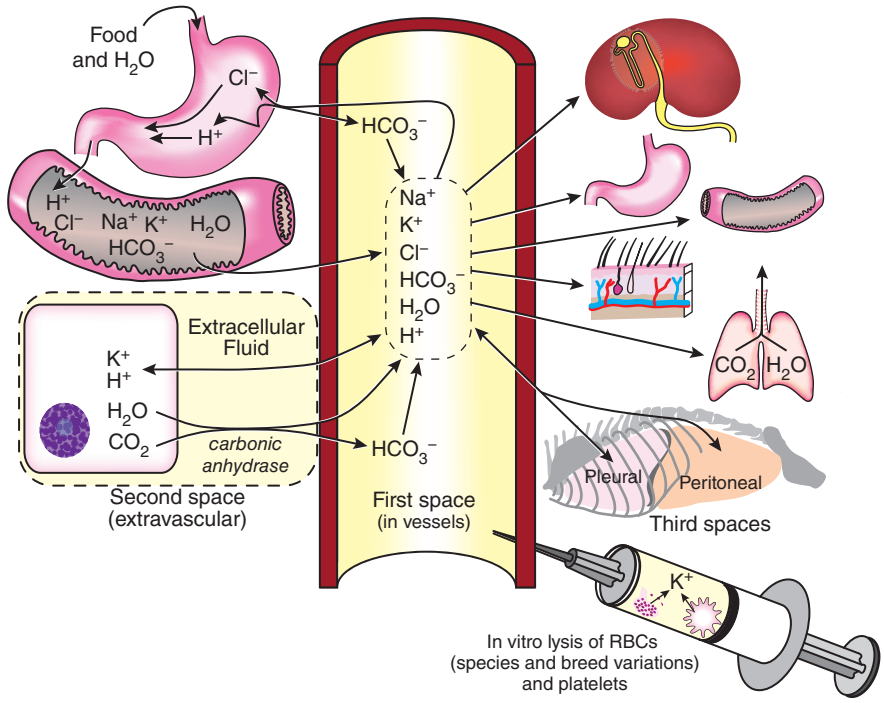


Figure 9.1. Basic concepts for electrolyte and H_2O movements into and out of plasma. Electrolytes and H_2O enter plasma (first space) from the alimentary tract, from cells, and by injections or infusions. In vitro lysis of blood cells and platelet activation may add K^+ to plasma or serum samples. Electrolytes and H_2O may leave the plasma and the body via kidneys, alimentary tract, skin, or respiratory tract, and they may enter extravascular sites within the body in the form of third-space loss (e.g., to pleural and peritoneal cavities). In some pathologic states involving muscle, H_2O or electrolytes shift between the ICF and extravascular ECF spaces (second space) (see the text for details). Fluids (e.g., plasma or urine) can enter pleural or peritoneal cavities and, if not removed via centesis, electrolytes and H_2O will be absorbed (see the text for details).

III. Electrolyte concentrations and acid-base abnormalities
   A. The regulation of acid-base status in an animal involves physiologic processes that may alter plasma concentrations of Na^+, Cl^-, K^+, and HCO_3^-, the major topics of this chapter.
   B. Pathologic states that first alter electrolyte concentrations can create acid-base abnormalities, the major topics of Chapter 10. Thus, it is difficult to completely separate electrolyte concepts from acid-base concepts. The early sections of Chapter 10 contain the major definitions and classifications of acid-base abnormalities. The end of Chapter 10 contains a section on SID. The SID approach uses electrolyte concentrations to define metabolic acid-base disorders.

SODIUM (Na^+) CONCENTRATION

I. Physiologic processes
   A. Plasma [Na^+] is nearly equivalent to ECF [Na^+], which is dependent on the ratio of tbNa^+ to tbH_2O (Eqs. 9.1a–c). Therefore, plasma [Na^+] must be interpreted with knowledge of

the patient's tbH_2O (i.e., state of hydration) and consideration of the patient's ECF volume (i.e., normovolemic, hypovolemic, or hypervolemic). Recognizing this simplified conceptual relationship assists in the understanding of most disorders that affect plasma $[Na^+]$.

$$\text{Normonatremia} \Rightarrow \text{normal } \frac{tbNa^+}{tbH_2O} \Rightarrow \frac{\text{normal}}{\text{normal}} \text{ or } \frac{\uparrow}{\uparrow} \text{ or } \frac{\downarrow}{\downarrow} \quad (\mathbf{9.1a})$$

$$\text{Hypernatremia} \Rightarrow \uparrow \frac{tbNa^+}{tbH_2O} \Rightarrow \frac{\uparrow}{\text{normal}} \text{ or } \frac{\text{normal}}{\downarrow} \text{ or } \frac{\downarrow}{\downarrow\downarrow} \quad (\mathbf{9.1b})$$

$$\text{Hyponatremia} \Rightarrow \downarrow \frac{tbNa^+}{tbH_2O} \Rightarrow \frac{\downarrow}{\text{normal}} \text{ or } \frac{\text{normal}}{\uparrow} \text{ or } \frac{\downarrow\downarrow}{\downarrow} \quad (\mathbf{9.1c})$$

$$\text{Plasma } [Na^+] \cong \frac{[Na_e^+] + [K_e^+]}{tbH_2O} \quad (\mathbf{9.1d})$$

where Na_e^+ and K_e^+ are the exchangeable and osmotically active pool of Na^+ and K^+

B. A more accurate relationship is shown in Eq. 9.1d, in which $Na^+{}_e$ and $K^+{}_e$ represent the exchangeable Na^+ and K^+ in the body (ions bound in molecules are not exchangeable).[1] From this equation, it can be seen that the total body exchangeable K^+ content influences plasma $[Na^+]$. When hypokalemia is present because of K^+ depletion, plasma $[Na^+]$ decreases. This change occurs as K^+ leaves cells and Na^+ enters to maintain electroneutrality (see Sodium Concentration, Section V.B.6). However, hyperkalemia tends not to cause a significant hypernatremia for two reasons: (i) marked hyperkalemia would be necessary to create changes in $[Na^+]$, but marked hyperkalemia is life-threatening, and (ii) increasing $[Na^+]$ stimulates thirst centers so that intake of H_2O reduces the magnitude of change.

C. Movement of Na^+ frequently drives movement of H_2O in response to Na^+-induced changes in osmotic pressure. Na^+ retention tends to cause H_2O retention (edema or ascites), and Na^+ wasting tends to cause loss of H_2O (hypovolemia or dehydration). However, H_2O does not follow Na^+ across renal collecting duct cell membranes in the absence of ADH, or across cell membranes of the ascending limb of the LoH.

D. Plasma $[Na^+]$ is controlled through two major mechanisms: regulation of blood volume and regulation of plasma osmolality.

1. Regulation of blood volume
   a. When hypovolemia or, more specifically, decreased effective blood volume is sensed by juxtaglomerular cells in kidneys, the RAAS is activated, which promotes formation of angiotensin II in the lungs and aldosterone in the adrenal cortices. Angiotensin II stimulates PCT resorption of Na^+, Cl^-, and H_2O. Aldosterone stimulates the active resorption of Na^+ in collecting ducts by opening Na^+ channels, enhancing Na^+-K^+-ATPase activity, and opening luminal K^+ channels. Its actions are probably mediated through aldosterone-induced proteins. Na^+-K^+-ATPase may increase because of increased production or decreased degradation (Figure 9.2).
   b. If hypovolemia is detected by carotid sinus baroreceptors, ADH is secreted, thus promoting renal H_2O resorption.
   c. If hypervolemia is sensed by atrial baroreceptors, Na^+ resorption is reduced through the actions of ANP to promote diuresis and natriuresis. ANP inhibits the RAAS, increases glomerular filtration rate and permeability, inhibits Na^+-K^+-ATPase activity

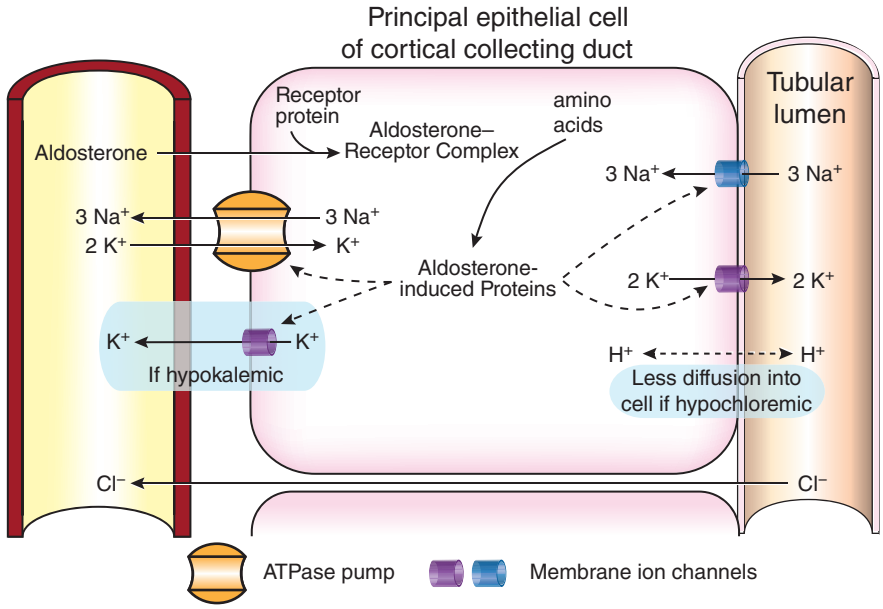


Figure 9.2. Actions of aldosterone on principal epithelial cells in cortical collecting ducts. Aldosterone enters the principal cells and binds to receptor proteins. The aldosterone-receptor complex stimulates the synthesis of aldosterone-induced proteins, which may include components of the Na^+-K^+-ATPase pump and membrane channels for Na^+ and K^+. Through the actions on the pump or channels, aldosterone promotes the following:

1. Na^+ is pumped from the cell to the peritubular fluid, and the resulting luminal cell gradient promotes the resorption of Na^+ through an opened apical Na^+ channel.
2. K^+ is pumped into the cell from the peritubular fluid and typically exits to the tubular fluid through an opened apical K^+ channel. When hypokalemia is present, K^+ may return to the peritubular fluid via an opened basolateral membrane channel (K^+ is recycled).
3. Typically, the Na^+-K^+-ATPase pump results in a net negative charge in the tubular fluid (three Na^+ resorbed and two K^+ secreted). The negative charge promotes the resorption of Cl^- through a paracellular route. When there is less luminal Cl^- available (as occurs with hypochloremia), the net negative charge promotes the retention of H^+ in the tubular fluid (the H^+ that passively enters and leaves the principal cells) and thus increases renal excretion of H^+.

The net result of aldosterone actions in health is the resorption of Na^+ and Cl^- and secretion of K^+ by principal cells. In the presence of hypochloremia, the electronegative gradient due to aldosterone activity indirectly increases excretion of H^+, which promotes aciduria. Aldosterone activity directly increases H^+ excretion via type A intercalated cells (Figure 9.4).

throughout the nephron, inhibits Na^+ transporters in the PCT, reduces activity of the Na^+-K^+-$2Cl^-$ cotransporter in the LoH, and most importantly, acts on the collecting ducts to decrease Na^+ channels and Na^+ resorption as well as ADH-dependent H_2O resorption.[2]

2. Regulation of plasma osmolality
   a. If hyperosmolality is sensed by hypothalamic osmoreceptors, thirst centers are stimulated to promote drinking of H_2O and ADH is released to promote H_2O resorption by the kidney. In a relatively minor role, ADH stimulates Na^+ and Cl^- resorption in the medullary thick limb of the LoH via actions on the Na^+-K^+-$2Cl^-$ carrier.

b. Conversely, hypoosmolality leads to decreased H_2O intake and increased urinary H_2O excretion due to decreased ADH secretion.

3. Plasma $[Na^+]$ is also somewhat self-regulated in that hyponatremia promotes aldosterone secretion and thus Na^+ retention, whereas hypernatremia inhibits aldosterone secretion and thus reduces Na^+ retention. However, plasma $[K^+]$ and alterations in effective blood volume are the major factors that control aldosterone secretion.[3]

E. Na^+ and Cl^- resorption in the DCT also involves an aldosterone-independent Na^+-Cl^- cotransporter; the resorption via this cotransporter increases with increased Na^+ delivery to the DCT. Thiazide diuretics block this cotransporter by binding to the Cl^- receptor.

F. The pathologic state of dehydration is equivalent to decreased tbH_2O.

1. Dehydrated animals have either a H_2O deficit or a H_2O and Na^+ deficit.
   a. Causes of a H_2O deficit without Na^+ deficit include decreased H_2O intake and loss of solute-poor H_2O (central diabetes insipidus, nephrogenic diabetes insipidus, or insensible respiratory losses). Respiratory loss of H_2O via water vapor is a pure H_2O loss. In the diabetes insipidus disorders, pure H_2O (i.e., osmolality = 0 mmol/kg) is not lost – there is always some solute in the urine and thus "solute-poor H_2O" is a more accurate expression.
   b. Causes of H_2O and Na^+ deficits include alimentary losses (vomiting, diarrhea, sequestration, or excessive salivation), renal losses (most polyuric states), and cutaneous losses (sweating).
2. Types of dehydration (from the perspective of the resulting body state)
   a. *Hypertonic:* hypernatremic, hyperosmolar dehydration caused by net hypoosmolar or hypotonic fluid loss, i.e., net H_2O loss > Na^+ loss
   b. *Isotonic:* normonatremic, isoosmolar dehydration caused by net isoosmolar or isotonic fluid loss, i.e., net H_2O loss $\cong$ Na^+ loss
   c. *Hypotonic:* hyponatremic, hypoosmolar dehydration caused by net hyperosmolar or hypertonic fluid loss, i.e., net H_2O loss < Na^+ loss

II. Analytical concepts

A. Assays, terms, and units

1. Ion-selective electrode assays are the most common for measuring $[Na^+]$ as the same sample can be used for most other chemical analytes in an automated instrument. Flame photometers for measuring $[Na^+]$ used to be the analytical gold standard, but the instruments are no longer used for routine patient samples.
2. Ion-selective electrode assays measure the electrical potential of Na^+ by direct or indirect potentiometry, and then the electrical potential is converted to concentration units.[4] Na^+ is mostly a free ion in plasma or serum H_2O (Figure 9.3).
   a. With direct potentiometry, Na^+ ion activity (electrical potential) is measured in a *nondiluted* sample by the interaction of Na^+ with the surface of an ion-selective electrode. The resultant $[Na^+]$ represents the $[Na^+]$ in the sample's H_2O, which is the $[Na^+]$ that drives physiologic or pathologic processes. If plasma is 93 % H_2O and the $[Na^+]$ in plasma H_2O is 161 mmol/L, then the plasma $[Na^+]$ is 150 mmol/L (161 mmol/L × 0.93 = 150 mmol/L). This "negative" difference is considered constant, and it results in an adjustment when direct potentiometry methods are calibrated.[4] This adjustment provides the plasma $[Na^+]$ (not the $[Na^+]$ in the aqueous phase) and assumes a plasma H_2O content of 93 %. If the sample contained proportionately less H_2O because of increased protein or lipid, the result would be the same because only the activity in the H_2O phase is measured. Similarly,

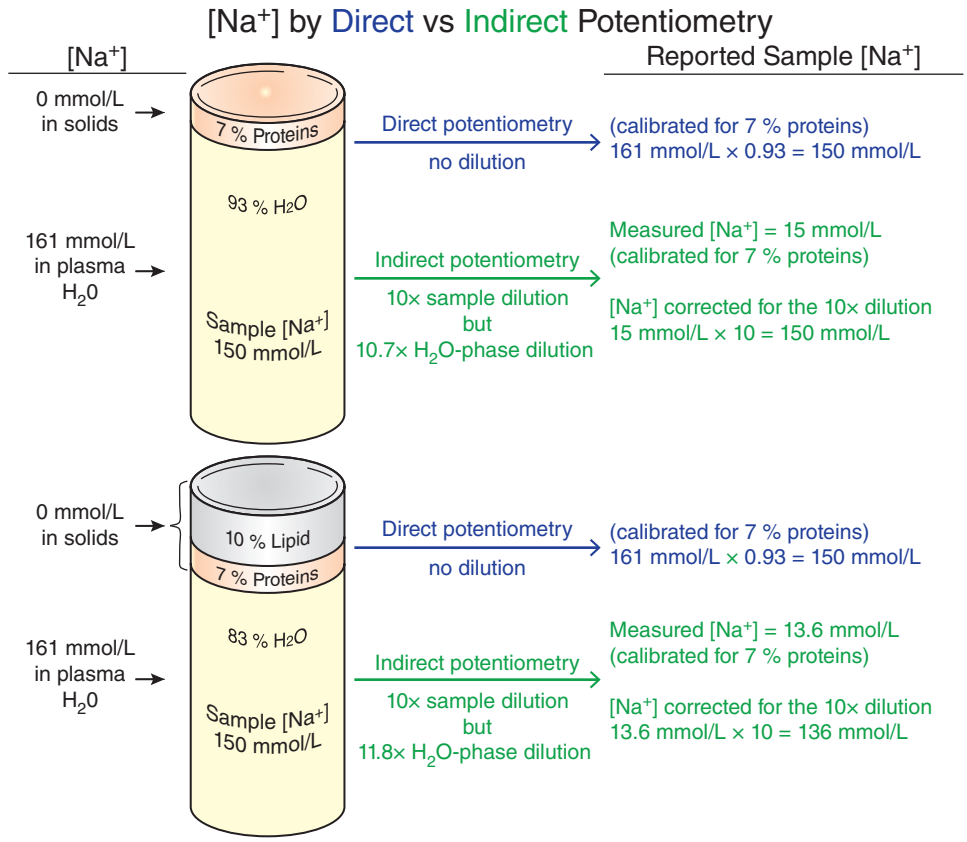


Figure 9.3. Electrolyte exclusion effect.
The body regulates the $[Na^+]$ in the aqueous portion of plasma (plasma H_2O). In the compared samples, the $[Na^+]$ in plasma H_2O of each is 161 mmol/L. Calculated values are approximate or rounded so the concepts can be seen.

In the top example, the plasma contains 7 % protein and 93 % H_2O (by volume). Na^+ is dissolved in the H_2O portion at 161 mmol/L; no Na^+ is in the volume occupied by proteins.

- By direct potentiometry, the method measures the $[Na^+]$ in the sample's H_2O (161 mmol/L) and then uses an assumed 93 % H_2O to calculate a reported sample concentration of 150 mmol/L.
- By indirect potentiometry, a small portion of the sample is diluted 1:10, which actually dilutes the $[Na^+]$ in the sample's H_2O by a little more than 1:10. The number of parts of H_2O in the final dilution per parts of H_2O in the sample aliquot = 9.93/0.93 = 10.7, making a 1:10.7 dilution. This yields 100 %/10.7 = 9.3 % of the original concentration in the sample's H_2O. The instrument measures a $[Na^+]$ in the diluted sample as 15 mmol/L (indirect potentiometry is calibrated to match direct potentiometry which was calibrated to match flame photometry). The instrument then calculates and reports a $[Na^+]$ of 150 mmol/L based on the 1:10 dilution of the sample. If the calculation were based on the actual 10.7× dilution of the aqueous phase, the $[Na^+]$ would be 15 mmol/L × 10.7 = 161 mmol/L.

In the bottom example, the plasma contains 10 % lipid, 7 % protein, and 83 % H_2O (by volume). Na^+ is dissolved in the H_2O portion at 161 mmol/L; no Na^+ is in the volume occupied by proteins and lipids.

- By direct potentiometry, the method measures the $[Na^+]$ in the sample's H_2O and then uses an assumed 93 % H_2O to calculate a reported sample concentration of 150 mmol/L.
- By indirect potentiometry, a small portion of the sample is diluted 1:10, which actually dilutes the $[Na^+]$ in the sample's H_2O by more than 10 %. The parts of H_2O in the final dilution per parts of H_2O in the sample aliquot = 9.83/0.83 = 11.8, making a 1:11.8 dilution. This yields 100 %/11.8 = 8.5 % of the original

hypoproteinemia would not impact the measurement. Note: The plasma H_2O content of two healthy cats and two healthy dogs were reportedly about 91 % and 91–92 %, respectively, not 93 %.[5] The mean serum H_2O content of 112 feline patients with various disorders was about 91 % (92 % in normoproteinemic cats).[6] Therefore, the standard adjustment factor may yield inaccurate values (1–2 mmol/L error for each 1 % difference in plasma H_2O) for at least some domestic mammals. However, hypernatremia and hyponatremia can still be detected because RIs are based on the same method and calculation. Note also that the H_2O content of serum and plasma may differ in health (comparison studies were not found) and more so in disease; marked hyperfibrinogenemia could decrease total serum H_2O content by about one percentage point.

b. With indirect potentiometry, the sample is *diluted* (e.g., 10 or 30 times) before Na^+ ion activity is measured, again by the interaction of Na^+ with the surface of an ion-selective electrode. The Na^+ ion activity measured depends on the $[Na^+]$ in the H_2O portion of the diluted sample. The sample $[Na^+]$ is determined by multiplying the measured $[Na^+]$ by the dilution factor. If the sample contains increased lipids or proteins and therefore a lower percentage of H_2O, the H_2O portion will be diluted more than usual by the added diluent. When the measured value is multiplied by the sample dilution factor (rather than by the H_2O-phase dilution factor), the result is a falsely decreased value for $[Na^+]$, and pseudohyponatremia if the effect is great enough. The error increases with increasing sample dilution, but the progressive changes are very small and of no additional clinical significance beyond a 1:10 dilution.

c. Comparative effects of increased plasma solids on the different methods used to measure $[Na^+]$

   (1) The direct potentiometry value is not affected by plasma H_2O displacement because the conversion factor from the H_2O concentration to plasma concentration is constant and the sample is not diluted prior to analysis.

   (2) The indirect potentiometry value is affected by plasma H_2O displacement because of the greater dilution of $[Na^+]$ when the sample has less H_2O. Formulas have been empirically derived in an attempt to adjust $[Na^+]$ for increased or decreased protein and lipid concentrations based on indirect

Figure 9.3. *continued*
concentration in the sample's H_2O instead of 9.3 % without hyperproteinemia. The instrument measures a $[Na^+]$ in the diluted sample as 13.6 mmol/L. The instrument then calculates and reports a $[Na^+]$ of 136 mmol/L based on the 1:10 instead of the actual 1:11.8 dilution of the sample, which, if it could be factored in, would yield the appropriate concentration: 13.6 × 11.8/10.7 × 10 = 150 mmol/L, where 11.8/10.7 reflects the additional dilution caused by the increased solids concentration and decreased aqueous phase.

As stated above, the $[Na^+]$ in plasma H_2O of each sample is 161 mmol/L and, via direct potentiometry, the reported plasma $[Na^+]$ is 150 mmol/L in each. However, via indirect potentiometry, the nonlipemic sample produced a $[Na^+]$ of 150 mmol/L and the lipemic sample produced a $[Na^+]$ of 136 mmol/L (pseudohyponatremia). The falsely low concentration occurs because the lipemic sample has less plasma H_2O (by volume) and causes a greater dilution of Na^+ in the aqueous phase than in the entire sample. The $[Na^+]$ is considered pseudohyponatremia because the Na^+ content and thus concentration in the sample is less but the $[Na^+]$ in the plasma H_2O (the concentration regulated by physiologic processes) has not changed. The dilution error increases nonlinearly with the degree of dilution, the error being similar with 1:10 or greater dilutions.

potentiometry. However, reported protein and lipid correction factors have varied and the actual change depends on analytical methods (for Na^+, total protein, cholesterol, or triglycerides) and probably the types of proteins and lipids present. Adjustment formulas developed in one laboratory may not be applicable to another.

(a) Based on the analysis of sera from 774 human patients, the mean change in [Na^+] was near 0.7 mmol/L for each 1 g/dL in total protein concentration.[7] For cats, the effect was nearly twice that, with substantial variation among samples.[6]

(b) One group found that human [Na^+] decreased by ~1 mmol/L for every 10 mmol/L increase in total lipid (triglyceride and cholesterol) concentration.[8]

(3) Flame photometric methods, like indirect potentiometric methods, may yield falsely decreased [Na^+] in samples with lipidemia or hyperproteinemia because [Na^+] is measured per volume of total sample rather than per volume of only the aqueous phase.

3. Unit conversion: mEq/L × 1 = mmol/L, and mg/dL ÷ 2.3 = mmol/L (SI unit)[9]

B. Sample for [Na^+] quantitation

1. Serum and heparinized plasma may be used. [Na^+] is stable for months if the sample does not dehydrate.
2. Plasma
   a. Na_2EDTA plasma should not be used because the anticoagulant's Na^+ would be in the plasma and falsely increase values.
   b. The amount of Na^+ in Na-heparin, including the NaCl in liquid Na-heparin solutions, is too small to cause clinically relevant changes in heparinized plasma [Na^+] if used as recommended. Based on one manufacturer's information, filling Na-heparin vacuum tubes to a proper blood volume increases the measured [Na^+] by 1–2 mmol/L. The increase might be greater with underfilled tubes, in samples from patients with erythrocytosis (less plasma per blood volume), or with excess heparin in a syringe.
   c. Underfilling blood gas syringes containing Li^+-heparin may falsely decrease whole blood [Na^+] measured by direct potentiometry (the typical method for so-called blood gas analyzers). Li^+-heparin at a concentration of 100 IU/mL of blood was reported to cause a negative bias of 2–3 mmol/L when syringes were filled to one-third capacity (1 mL in a 3 mL syringe).[10] The decrease doubled when only 0.5 mL was collected. Decreases may be caused by Na^+ binding to heparin, which is negatively charged, or effects of Li^+-heparin on the function of at least some ion-selective electrodes.
3. For species that have relatively Na^+-poor RBCs (e.g., ~15 mmol/L in horses, pigs, and people),[11] dilution caused by in vitro hemolysis can reduce the measured [Na^+].

III. Hypernatremia

A. Hypernatremia occurs primarily when the ratio of $tbNa^+$ to tbH_2O is increased (Eq. 9.1b), but also with the shifting of H_2O from ECF to ICF.

B. Disorders and pathogeneses (Table 9.2)

1. H_2O-deficit group (decreased tbH_2O, hypertonic dehydration from hypotonic fluid loss): Dehydration may cause hypernatremia directly via hemoconcentration (pure H_2O loss) and indirectly by activating the RAAS, which stimulates renal Na^+ retention via actions of aldosterone and angiotensin II. In most nephron segments, Na^+ resorption

Table 9.2. Disorders and conditions that cause hypernatremia
H_2O-deficit group (decreased tbH_2O, hypertonic dehydration)
Inadequate H_2O intake
H_2O deprivation*
Defective thirst response (hypothalamic defect)
Solute-poor H_2O loss (without adequate H_2O replacement)
Insensible loss:* panting, hyperventilation, or fever
Diabetes insipidus (central or nephrogenic)
Adrenocortical neoplasm secreting corticosterone and aldosterone
Osmosis causing H_2O loss > Na^+ loss
Renal loss: osmotic diuresis*
Alimentary loss: osmotic diarrhea, osmotic sequestration, PO_4 enemas, ruminal acidosis, or paintball toxicosis
Na^+-excess group (increased tbNa^+)
Excess Na^+ with concurrently restricted H_2O intake
Salt poisoning
Administration of hypertonic saline or sodium bicarbonate
Decreased renal excretion of sodium: hyperaldosteronism
Reset or defective osmoreceptor (essential hypernatremia)
Other or unknown mechanism
Severe exercise in racing greyhounds
Sample dehydration or sublimation
Pseudohypernatremia due to marked hypoproteinemia

* A relatively common disorder, condition, or mechanism

promotes H_2O resorption, but because collecting ducts are permeable to H_2O only in the presence of ADH, Na^+ retention without sufficient ADH activity results in hypernatremia.

a. Inadequate H_2O intake

(1) H_2O deprivation: Access to H_2O may be restricted because of accidents (water bowl turned over on a hot day), weather (frozen water tank), or other situations. Without H_2O intake, physiologic H_2O losses via kidneys, lungs, skin, or intestine may produce dehydration.

(2) Defective thirst response (hypodipsic or adipsic hypernatremia): Hypothalamic disease (trauma, neoplasms, inflammation, developmental anomalies) may damage the osmoreceptor that triggers a thirst response, or the thirst center itself may be damaged. Hypodipsic hypernatremia has been reported in cats and dogs, particularly in young ones with lobar holoprosencephaly.[12,13] Some cases have been considered "essential hypernatremia," a term applied to people who seem to have an upward shift in the setpoints for thirst and osmoregulation characterized by (i) sustained hypernatremia not corrected by fluid administration, (ii) little or no evidence of decreased ECF volume, (iii) appropriate ADH secretion in response to hypovolemia (but not to hypertonicity), and (iv) an appropriate renal tubular response to ADH.[14-16] However, dehydration has been present in most reports of hypodipsic hyponatremia in dogs and cats.[12,17,18]

b. Solute-poor H_2O loss without H_2O replacement
   (1) Insensible loss of H_2O by panting, hyperventilation, or fever: The animal becomes H_2O depleted by losing H_2O via the respiratory system or skin.
   (2) Diabetes insipidus (central or nephrogenic) (see Chapter 8 for disorders)
      (a) In the diabetes insipidus disorders, diminished ADH activity or response in the collecting ducts may result in solute-poor H_2O loss, i.e., urine with very low concentrations of Na^+ and other solutes, and therefore a urine specific gravity nearing 1.000.
      (b) Animals with diabetes insipidus that have unrestricted access to H_2O may drink sufficiently to prevent hypernatremia.
   (3) Adrenocortical neoplasm secreting corticosterone and aldosterone in a dog[19]
      (a) This disorder is uncommon. The laboratory features of the case were hypernatremia (marked), hyperchloremia, hypokalemia, hyposthenuria, and abnormal results of adrenocorticotropic hormone stimulation tests: inadequate increase in plasma [cortisol], exaggerated increase in [corticosterone], and exaggerated increase in [aldosterone].
      (b) Given that most patients with hyperaldosteronism are normonatremic and hypernatremia is typically mild if present, this marked hypernatremia might have related to renal loss of solute-poor H_2O because of the inhibition of ADH release by corticosterone.

c. Osmosis causing H_2O loss > Na^+ loss: During osmosis not caused by [Na^+] gradients, the H_2O that moves toward greater solute concentration contains a small amount of Na^+ because of solute drag, but the net result is a greater loss of H_2O than Na^+, and thus hypernatremia occurs. Unrestricted access to H_2O may prevent this hypernatremia. Also, other processes in such cases (e.g., ketosis in the case of diabetes mellitus with osmotic diuresis from glucosuria) may increase renal Na^+ loss and counteract hypernatremic tendencies.
   (1) Renal loss: Osmotic diuretic agents (e.g., glucose and mannitol) in renal tubular fluid inhibit passive H_2O resorption.
   (2) Alimentary loss
      (a) Accumulation of osmotic agents, as occurs in some diarrheas or with use of activated charcoal (± sorbitol, glycerol, or propylene glycol), inhibits intestinal H_2O absorption.
      (b) A PO_4 enema pulls H_2O from ECF spaces to the colon. Concurrent colonic absorption of Na^+ may augment the hypernatremia.[20,21] Concurrently, marked hyperphosphatemia and hypocalcemia may occur (see Chapter 11).
      (c) In ruminal acidosis (grain overload), accumulation of solutes (including lactate) in the rumen causes the osmotic movement of H_2O into the rumen, thus producing hypernatremia.[22]
      (d) Dogs with paintball toxicosis (after ingestion of paintballs) may develop hypernatremia because the paintballs contain osmotic molecules (e.g., glycerol and sorbitol) that pull H_2O from plasma to intestines.[23]

2. Na^+-excess group (increased $tbNa^+$)
   a. Excess Na^+ intake with concurrent restricted H_2O intake (rare)
      (1) Salt poisoning: Cattle with excessive Na^+ (and Cl^-) intake and with concurrent restricted access to H_2O may develop an increased $tbNa^+$ (and total body Cl^- content) and thus hypernatremia (and hyperchloremia).[24] Extreme

hypernatremia and hyperchloremia occurred in a dog that ingested a salt–flour mixture that was used as modeling clay.[25]

(2) Administration of hypertonic saline or sodium bicarbonate can lead to increased $tbNa^+$ and thus hypernatremia (and hyperchloremia or increased $[HCO_3^-]$).

b. Decreased renal excretion of Na^+: hyperaldosteronism

(1) Excessive aldosterone promotes excessive renal Na^+ (and Cl^-) retention. Hypernatremia (and hyperchloremia) may occur if there is H_2O restriction or defective ADH activity.

(2) However, because of a mechanism referred to as aldosterone escape, hyperaldosteronism does not typically cause hypernatremia. Once Na^+ retention begins and there is corresponding H_2O retention, natriuresis prevents the development of hypernatremia. Hypervolemia-induced increases in renal perfusion pressure and ANP likely contribute to the increased natriuresis, the former by increasing renal tubular flow rates, decreasing proximal Na^+ resorption, and overwhelming Na^+ resorption capacity in the distal nephron. ANP inhibits Na^+ resorption in the collecting duct. Normonatremia was found in 12 of 13 cats reported to have primary hyperaldosteronism; the thirteenth cat was hypernatremic.[26]

c. Reset or defective osmoreceptor

(1) This may occur in what has been called essential hypernatremia (see preceding Section III.B.1.a), a disorder in which there is persistent hypernatremia with lack of ADH and appropriate thirst responses to hyperosmolality, but without loss of the ability to form concentrated urine. Although affected human patients may have mild subclinical hypovolemia or dehydration,[15,27] dehydration and hypovolemia are not clinically apparent, thus suggesting an increase in $tbNa^+$ rather than just impaired H_2O intake and resorption. Evidence suggests that some of these patients have excessive salt intake because autoantibodies to sensory circumventricular organs impair regulation of salt intake.[16]

(2) A diagnosis of essential hypernatremia in a young dog was based on finding hypernatremia, hyperchloremia, adipsia, and ability to concentrate urine only when hypovolemic; [aldosterone] and $[K^+]$ were WRIs.[18]

3. Other or unknown mechanisms

a. Severe exercise in racing greyhounds:[28–30] During and immediately after a race, the $[Na^+]$ in plasma may be 10–20 mmol/L above prerace concentrations. If there is concurrent hypovolemia and lactic acidosis, the hypernatremia may be caused by the shifting of H_2O from ECF to ICF. The accumulation of L-lactate in muscle fibers may create the osmotic gradient.[1]

b. Sample dehydration or sublimation: Exposure of serum or plasma to air may allow evaporation that causes spurious hypernatremia. This is especially true when air-conditioning systems blow cool, dry air over the sample processing or analysis areas. Sublimation of H_2O from frozen samples may increase the $[Na^+]$ if the storage container is not adequately sealed or if it is too large for the sample size.

c. Pseudohypernatremia due to marked hypoproteinemia: As explained with analytical concepts above, pseudohyponatremia may occur when high protein or lipid content in a serum or plasma sample displaces plasma H_2O and the sample is analyzed by indirect potentiometry (or flame photometry). Conversely, pseudohypernatremia

may be present when the plasma or serum sample has a marked hypoproteinemia and thus a greater H_2O content.[7,31] The change in [Na^+] for each g/dL change in protein is expected to be < 2 mmol/L, the increment varying with types of proteins and variations in methods for indirect potentiometry and total protein concentration.

IV. Normonatremia in dehydrated or edematous animals
  A. Normonatremia does not necessarily indicate that the Na^+ balance is normal (Eq. 9.1a). Recognizing the possibility of altered Na^+ regulation in normonatremic animals is important in patient management. That is, do you need to administer Na^+ or restrict Na^+ intake?
  B. Disorders and pathogeneses (Table 9.3)
    1. Net loss of isotonic fluids that causes dehydration
      a. Alimentary loss of isotonic fluid may occur with vomiting, diarrhea, or sequestration.
      b. Renal loss of Na^+ and H_2O may occur in several situations:
        (1) Many polyuric renal diseases cause Na^+ and H_2O loss because of defective tubular functions.
        (2) Osmotic diuresis impairs resorption of H_2O in tubules. A high tubular flow rate also contributes to Na^+ loss.
        (3) Most diuretic agents (e.g., furosemide or thiazides) cause Na^+ and H_2O loss through interference with Cl^- resorption.
      c. Cutaneous loss from horses: The [Na^+] in equine sweat has been reported to be mildly lower or higher than the [Na^+] in serum or plasma, varying with anatomic site, stimulus for sweating (e.g., epinephrine versus heat), and other factors including method of assessment.[32–35] Profuse sweating without increased H_2O intake could cause normonatremic dehydration.
    2. Net retention of isotonic fluids that causes edema or transudate (Note: Edematous disorders may create either normonatremia or hyponatremia, depending on the relative retention of Na^+ and H_2O.)
      a. Congestive heart failure (caused by valvular disease or cardiomyopathies)
        (1) Decreased cardiac output and stroke volume lead to (i) impaired sensitivity of baroreceptors, thus reducing parasympathetic signals and increasing

Table 9.3. Disorders and conditions that cause normonatremia in dehydrated or edematous animals

Net loss of isotonic fluids causing dehydration
Alimentary loss: vomiting, diarrhea,* sequestration*
Renal loss: renal disease,* osmotic diuresis,* diuretics
Cutaneous loss from horses: sweating*
Net retention of isotonic fluids causing edema or transudate
Congestive heart failure*
Hepatic cirrhosis*
Nephrotic syndrome*

* A relatively common disorder, condition, or mechanism

sympathetic stimulation of nonosmotic ADH release, and (ii) release of aldosterone via the RAAS.[36,37] The renal response to ADH is retention of solute-free H_2O. The renal response to aldosterone is the retention of Na^+.

(2) The presenting edematous animal may be normonatremic or hyponatremic. When ADH plays a major role, hyponatremia occurs because there is greater H_2O retention than Na^+ retention.

b. Hepatic cirrhosis with abdominal transudation

(1) Presinusoidal portal hypertension occurs because of two major hormonal changes: (i) nonosmotic stimulation of ADH release and (ii) stimulation of the RAAS by decreased effective blood volume.[38–41] The periportal fibrosis in canine cirrhosis may restrict portal blood flow and thus contribute to portal hypertension.

(a) Systemic vasodilation may be due to several vasodilators including nitrous oxide. The effect includes altered splanchnic arterial circulation, which leads to blood-pooling and ineffective blood volume. Activation of baroreceptors leads to the hypothalamic release of ADH and subsequent solute-free H_2O retention by kidneys.

(b) The ineffective blood volume also stimulates the RAAS, which leads to aldosterone release and subsequent Na^+ retention by the renal collecting ducts.

(2) The presenting edematous animal may be normonatremic or hyponatremic. The effect of ADH plays a major role in cirrhosis; hyponatremia occurs when there is greater H_2O retention than Na^+ retention.

(3) Hypoalbuminemia and hypoproteinemia play secondary roles in ascites formation.

(a) In health, plasma proteins create colloid osmotic (oncotic) pressure (about 80 % from albumin and 20 % from globulins) that helps retain H_2O in vessels that are relatively impermeable to proteins. When hypoproteinemia is present with hepatic cirrhosis, the increases in vascular hydraulic pressure have more effect than hypoproteinemia in promoting movement of H_2O from some vessels (hepatic or pulmonary) to extravascular spaces.

(b) Hypoalbuminemia alone will not cause the transudation. People with analbuminemia may have mild dependent edema but do not have generalized transudation; this is because of compensatory processes (increased [globulins], altered capillary hydraulic pressure, or altered renal blood flow).[42,43] Also, hepatic sinusoids are freely permeable to albumin and H_2O, so plasma H_2O moves out of the sinusoids in either the presence or the absence of hypoalbuminemia.

c. Nephrotic syndrome (protein-losing nephropathy that leads to cavitary transudation and edema)[1,44]

(1) The pathogenesis of Na^+ and H_2O retention involves several processes including increased activity of the RAAS, nonosmotic stimulation of ADH release, decreased renal responsiveness to ANP, decreased plasma protein concentration, inappropriate neural reflexes involving the kidneys, and glomerular disease.

(2) If the renal disease also causes loss of tubular functions, the kidneys have less ability to retain Na^+ and H_2O, and thus the edematous state may not develop in some cases of protein-losing nephropathy.

V. Hyponatremia
   A. Hyponatremia typically occurs when the ratio of $tbNa^+$ to tbH_2O is decreased (Eq. 9.1c) or with the shifting of H_2O from ICF to ECF.
   B. Disorders and pathogeneses (Table 9.4)
      1. Na^+-deficit group: net Na^+ loss > H_2O loss (hypotonic dehydration). In the following disorders, the $[Na^+]$ in lost fluid is usually not greater than the plasma $[Na^+]$, but loss of Na^+-containing fluid followed by drinking of H_2O may cause hyponatremia.
         a. Alimentary loss of Na^+-containing H_2O may occur with vomiting, diarrhea, sequestration, excess salivation, canine whipworm infections, and bovine hemorrhagic bowel syndrome.[45] For these conditions to cause hyponatremia, there probably is a loss of isotonic ECF followed by drinking H_2O and renal H_2O retention (ADH response) that dilute the remaining plasma Na^+.
         b. Renal loss
            (1) Hypoadrenocorticism:[1] Adrenal insufficiency decreases the [aldosterone], thus causing less resorption of Na^+ and subsequently Cl^- by the renal principal cells.

Table 9.4. Disorders and conditions that cause hyponatremia

Na^+-deficit group: net Na^+ loss > H_2O loss (loss of Na^+-containing fluid followed by increased H_2O intake, hypotonic dehydration)
Alimentary loss: vomiting, diarrhea,* sequestration,* canine whipworm infection, excess salivation, bovine hemorrhagic bowel syndrome
Renal loss: hypoadrenocorticism,* prolonged diuresis,* thiazide diuretics, ketonuria,* Na^+-wasting nephropathies, selective hypoaldosteronism (without hypocortisolemia)
Cutaneous loss: sweating in horses*
Third-space loss: repeated drainage of chylous effusion, acute hemorrhage or exudation
H_2O-excess groups (H_2O retention > Na^+ retention) with or without edema
Edematous disorders
Congestive heart failure*
Hepatic cirrhosis
Nephrotic syndrome
Heat stress in camelids
Expanded ECF volume (but without edema)
Syndrome of inappropriate ADH secretion
Excess administration of Na^+-poor fluids
Primary polydipsia
Shifting of H_2O from ICF to ECF
Hyperglycemia*
Mannitol infusion (IV)
Shifting of Na^+ from ECF to ICF: acute muscle damage
Shifting of Na^+ from intravascular to extravascular fluid: uroperitoneum
K^+ depletion causing shifts in Na^+ and H_2O
Pulmonary disease
Pseudohyponatremia due to hyperlipidemia or hyperproteinemia

* A relatively common disorder, condition, or mechanism

Decreased Na^+ and Cl^- resorption leads to decreased plasma osmolality and decreased renal medullary hypertonicity. The latter leads to a decreased ability to resorb H_2O, so hypovolemia ensues. Hypovolemia stimulates ADH release and thirst centers. Increased ADH activity and increased H_2O intake dilute ECF Na^+ and thus cause hyponatremia (and hypochloremia). Hypocortisolemia promotes the hypothalamic release of ADH (multiple theories), which causes defective excretion of H_2O (retention of solute-poor H_2O in the presence of hypoosmolality) and a dilutional hyponatremia.[1,46] In people with hypoaldosteronism but lack of hypocortisolemia, normonatremia is maintained by enhanced tubular resorption of Na^+ caused by increased angiotensin II, decreased ANP, and enhanced passive resorption.[1]

(2) Prolonged diuresis
   (a) Prolonged osmotic diuresis caused by administration of Na^+-poor fluid (e.g., 5 % dextrose)[47] or other forms of diuresis (e.g., caused by furosemide administration)[48] tend to deplete Na^+ and H_2O, cause hypovolemia, and stimulate ADH release and thirst centers. H_2O intake tends to dilute the Na^+ in the ECF and thus cause hyponatremia.
   (b) Thiazide diuretics cause Na^+, Cl^-, and K^+ loss in excess of H_2O loss, and thus hyponatremia, hypochloremia, and hypokalemia may develop. Urinary Na^+ and Cl^- loss is increased because thiazides inhibit a Na^+-Cl^- cotransporter in DCTs. Urinary K^+ loss is increased because of an increased flow rate in collecting ducts and hypovolemia-induced release of aldosterone, which opens K^+ channels for excretion. The hypovolemia also stimulates ADH release, which promotes resorption of H_2O and thus dilution of the remaining Na^+, Cl^-, and K^+.

(3) Ketonuria
   (a) During ketonuria, the excretion of ketone bodies (AcAc and BHB) is increased. The presence of these anions in the tubular lumen obligates the excretion of cations, and thus there is increased renal excretion of Na^+.
   (b) Concurrent osmotic diuresis (from glucosuria) may compound the Na^+ loss associated with ketonuria in patients with diabetes mellitus (see the preceding entry regarding prolonged osmotic diuresis for factors that may alter $[Na^+]$).

(4) Na^+-wasting nephropathies: Some renal diseases (especially tubular diseases or pyelonephritis) cause an excess excretion of Na^+ because of decreased Na^+ resorption. This is seen more in horses than other domestic mammals.

(5) Hyperreninemic hypoaldosteronism (see hyperkalemia)

c. Cutaneous loss by sweating
   (1) Among the domestic mammals, only horses sweat sufficiently to cause electrolyte and H_2O imbalances.
   (2) Equine sweat is a K^+- and Cl^--rich fluid (i.e., concentrations are greater than plasma concentrations) with some reports indicating it may also be rich in Na^+ (evaporation might contribute to measured increases).[32–35] Therefore, sweating might cause hyponatremia from hypertonic fluid loss, and drinking of H_2O or the ADH-stimulated retention of H_2O after sweating may lead to dilutional hyponatremia.

d. Third-space loss (loss to pleural or peritoneal cavity)
   (1) Repeated drainage of chylous thoracic effusions

(a) Repeated removal of isotonic fluid from the thoracic cavity probably results in a Na^+- and H_2O-depleted state that is followed by intake of H_2O and an ADH response to cause dilutional hyponatremia.[49,50]

(b) This type of hyponatremia would probably also result from repeated removal of other cavitary effusions, but such removal is not as common as it is for chylous effusions.

(2) Acute internal hemorrhage or acute exudation

(a) Acute loss of ECF from the vascular space to other extracellular sites in the body (e.g., third-space loss) causes isotonic hypovolemia. If followed by increased intake of H_2O and an ADH response, dilutional hyponatremia may develop.

(b) This type of hyponatremia is rare, since hypovolemia would also stimulate renal Na^+ retention.

2. H_2O-excess group (H_2O retention > Na^+ retention) with or without edema

a. Edematous disorders

(1) Congestive heart failure, hepatic cirrhosis, and nephrotic syndrome may expand ECF volume and cause edema. Although these conditions are often associated with normonatremia (see the preceding Section IV.B.2), hyponatremia (typically mild) may occur if the ratio of $tbNa^+$ to tbH_2O is decreased.

(2) Heat stress in camelids: Hyponatremia is found in llamas and alpacas during heat stress, which is seen in warmer climates, more commonly in unsheared than in sheared alpacas.[51] Concurrent scrotal edema, hypoproteinemia, and hypoalbuminemia support the conclusion of increased tbH_2O. However, lack of a concurrent magnitude of hypochloremia indicates that other factors contribute to the hyponatremia.

b. Expanded ECF volume (but without edema)

(1) Syndrome of inappropriate ADH secretion (SIADH)

(a) This is characterized by nonphysiologic release of ADH in the presence of hypoosmolality and hypervolemia. Excess ADH with unrestricted H_2O intake leads to solute-poor H_2O resorption and dilutional hyponatremia. Renal Na^+ excretion (perhaps due to ANP) is increased because of activation of volume receptors.[1]

(b) Neurologic (inflammatory, neoplastic, traumatic, developmental), pulmonary, and thyroid disorders are associated with the syndrome of inappropriate ADH secretion, as are many drugs. A few cases have been reported in domestic mammals.[52–56]

(2) Excessive administration of Na^+-poor fluids: Administration of excess oral water or IV 5 % dextrose or 0.45 % saline could lead to dilutional hyponatremia.[57]

(3) Primary polydipsia may cause hyponatremia, but it is typically a minor abnormality because functional kidneys can excrete the consumed H_2O.[58]

3. Shifting of H_2O from ICF to ECF

a. Hyperglycemia or mannitol infusion: A marked or persistent increase in effective plasma osmolality (e.g., because of hyperglycemia or mannitol infusion) creates an osmolal (and osmolar) gradient between ECF and ICF. This draws H_2O into the blood by osmosis, thus diluting the plasma [Na^+]. In theory, a glucose-adjusted [Na^+] can be calculated that would represent the [Na^+] if the hyperglycemia were not present.

(1) A frequently cited adjustment factor of 1.6 mmol/L for each increase of 100 mg/dL of glucose was derived for a 70 kg person with a baseline [Na^+] of 140 mmol/L and a [glucose] of 100 mg/dL.[59] Application of this factor is shown in Eq. 9.2a. Although usually called a *correction factor* for a *corrected [Na^+]*, implying incorrect measurement of [Na^+] when hyperglycemia is present, such [Na^+] measurements are correct; they are just partially the result of hyperglycemia. Appropriately adjusting for the effects of hyperglycemia on [Na^+] may allow recognition of other factors concurrently affecting [Na^+].

(2) When hyperglycemia was created in six healthy people, it was found that the hyperglycemic effect on [Na^+] was not linear.[60] The adjustment factor of 1.6 was considered appropriate when [glucose] was ≤ 400 mg/dL (Eq. 9.2a), but an adjustment factor of 4 was needed for [glucose] > 400 mg/dL (Eq. 9.2b). The average effect was 2.4 mmol/L for every 100 mg/dL increase in [glucose].

(3) The osmotic effect of hyperglycemia to reduce plasma [Na^+] is expected to be similar among mammals.

$$\text{Glucose-adjusted } [Na^+] = \text{patient's } [Na^+] + \left(\frac{\text{patient's [glucose]} - \text{URL}}{100 \text{ mg/dL}} \times 1.6 \text{ mmol/L}\right)$$

Example: Patient's data: [Na^+] = 130 mmol/L, [glucose] = 620 mg/dL

Glucose RI = 80–120 mg/dL, so the URL (upper reference limit) = 120 mg/dL

$$\text{Glucose-adjusted } [Na^+] = 130 \text{ mmol/L} + \left(\frac{620 - 120 \text{ mg/mL}}{100 \text{ mg/dL}} \times 1.6 \text{ mmol/L}\right)$$

$$\text{Glucose-adjusted } [Na^+] = 138 \text{ mmol/L}$$

(**9.2a**)

For patient's [glucose] > 400 mg/dL:

$$\text{Glucose-adjusted } [Na^+] = \text{patient's } [Na^+] + \left(\frac{\text{patient's [glucose]} - \text{URL}}{100 \text{ mg/dL}} \times 4 \text{ mmol/L}\right)$$

Example: Patient's data: [Na^+] = 130 mmol/L, [glucose] = 620 mg/dL

Glucose RI = 80–120 mg/dL, so the URL = 120 mg/dL

$$\text{Glucose-adjusted } [Na^+] = 130 \text{ mmol/L} + \left(\frac{620 - 120 \text{ mg/mL}}{100 \text{ mg/dL}} \times 4 \text{ mmol/L}\right)$$

$$\text{Glucose-adjusted } [Na^+] = 150 \text{ mmol/L}$$

(**9.2b**)

b. Concurrent processes (osmotic diuresis and ketonuria) may be present and also contribute to renal Na^+ loss, decreased $tbNa^+$, and thus hyponatremia. Also, drinking, stimulated by hyperosmolality (due to hyperglycemia) or hypovolemia (excessive renal loss of H_2O), can contribute to dilution of the [Na^+].

4. Shifting of Na^+ from ECF to ICF: acute muscle damage (various causes of rhabdomyolysis that may also cause hyperkalemia) (Table 9.5)
   a. Acute damage to cell membranes of muscle fibers allows Na^+ to move from a high concentration in ECF to a lower concentration in ICF.[61]

b. In this condition, there is a concurrent influx of H_2O and fCa^{2+} and efflux of K^+ and PO_4 to cause hypovolemia, hypocalcemia, hyperkalemia, and hyperphosphatemia (a pattern that could also suggest a ruptured bladder).

5. Shift of Na^+ from intravascular to extravascular space: uroperitoneum
   a. When urine, which is poor in Na^+ and Cl^-, is in the abdominal cavity (*uroperitoneum*), diffusion of Na^+ and Cl^- from the plasma to the peritoneal fluid may cause hyponatremia and hypochloremia.[62]
   b. Experimentally, in dogs, hyponatremia and hypochloremia were detected 1–2 d after the onset of uroperitoneum, and they were marked after 4 d.[63]
6. K^+ depletion causing shifts in Na^+ and H_2O[64,65] (Eq. 9.1d)
   a. As described later in this chapter (see Potassium Concentration, Section V.B.2), K^+ can be lost from the body during a variety of gastrointestinal and renal disorders. If these losses are not replaced by intake, the body becomes K^+ depleted.
   b. These K^+ losses decrease the extracellular $[K^+]$, which creates a gradient for K^+ movement from cells to the ECF. Extracellular shifting of K^+ obligates an increase in extracellular H_2O which dilutes the ECF Na^+. For electroneutrality, Cl^- likely leaves cells with K^+ (and may then exchange with intracellular HCO_3^-), also lowering intracellular osmolality and promoting H_2O movement from cells to ECF, thus further diluting the ECF Na^+ and decreasing the plasma $[Na^+]$.
7. Horses with septic pulmonary disease with or without pleural effusion are commonly hyponatremic.[66] One potential cause is inappropriate stimulation of ADH, but this is unproven.
8. Pseudohyponatremia due to hyperlipidemia or hyperproteinemia
   a. This may occur when the serum or plasma $[Na^+]$ is determined by flame photometry or indirect potentiometry (see Sodium Concentration, Section II.A.2 and Figure 9.3). If measured by indirect potentiometry, the same principles apply to $[Cl^-]$ and pseudohypochloremia.
   b. Usually, the hyperlipidemia that creates pseudohyponatremia occurs when a serum or plasma sample has hypertriglyceridemia and/or visual lipemia associated with increased concentrations of chylomicrons or very-low-density lipoproteins (see Chapter 16 for disorders or conditions). However, this pseudohyponatremia has also been recognized in people with hypercholesterolemia due to accumulation of lipoprotein X in plasma; visual lipemia or hypertriglyceridemia was not present.[67–69] In these reported cases, the hypercholesterolemia was associated with hepatic disease or cholestasis. Increased concentrations of lipoprotein X can cause pseudohyponatremia because the molecules are as large as or larger than very-low-density lipoproteins and thus can significantly displace water within a plasma or serum sample.[68]
   c. When hyperproteinemia creates pseudohyponatremia, there typically is marked hyperproteinemia (> 10 g/dL) that may be associated with paraproteinemia of a lymphoid neoplasm.
   d. Although indirect potentiometry is the usual method used for measuring $[Na^+]$ as part of routine chemistry panels at reference laboratories, if serum or plasma $[Na^+]$ is measured by direct potentiometry (no sample dilution), excess solids in the plasma do not affect results.

VI. Disorders causing hyponatremia or normonatremia

A. Several disorders alter $tbNa^+$ and tbH_2O status so that an animal with the disorder may be either hyponatremic or normonatremic. As noted at the beginning of this section, the

plasma [Na^+] is highly dependent on the ratio of $tbNa^+$ to tbH_2O. Accordingly, hyponatremia will be found when this ratio is decreased, but normonatremia will be present if the ratio is not significantly changed.

1. Most disorders that lead to dehydration (thus decreased tbH_2O) have a concurrent loss of Na^+ and H_2O (e.g., alimentary loss, renal loss, cutaneous loss) and result in a normonatremic dehydration. However, if the animal has access to H_2O and/or there is renal conservation of H_2O but not corresponding Na^+ retention, then the animal may be hyponatremic instead of normonatremic. It is very unlikely that lost fluid would have a [Na^+] greater than the plasma [Na^+].
2. With most disorders that lead to generalized edema and/or transudation (thus increased tbH_2O), there is a concurrent retention of Na^+ and H_2O. Depending on the relative amounts of Na^+ and H_2O retention, there can be either normonatremia or hyponatremia.

B. When polyuria is due to osmotic diuresis, multiple factors and possibly concurrent conditions may produce normonatremia or hyponatremia. Relevant historical and current information about an animal should be considered when interpreting [Na^+] (and [Cl^-]) in these patients.

1. Glucosuria (or other conditions of osmotic diuresis) by itself only promotes the loss of H_2O, thus plasma [Na^+] and [Cl^-] tend to increase. If dehydration stimulates thirst, then enough H_2O may be consumed to balance the H_2O loss and thus normonatremia and normochloremia may be present. Concurrently, hypovolemia may stimulate the RAAS to promote aldosterone-dependent conservation of Na^+ and Cl^- and concurrently stimulate release of ADH to promote renal conservation of H_2O. When glucosuria is due to marked hyperglycemia, osmosis promotes shifting of H_2O from interstitial and ICF to intravascular fluid leading to hyponatremia; marked hyperglycemia can contribute to hyperosmolal plasma and thus a stimulus for ADH release and thirst, also contributing to hyponatremia.
2. With osmotic diuresis, the higher flow rate in the renal tubules leads to less time for passive resorption of Na^+ and, secondarily, Cl^-. Thus, normonatremia and normochloremia may be present in the dehydrated animal.
3. Concurrent excretion of the anions of ketonuria obligate the renal excretion of cations including Na^+. If there is less Na^+ resorption, there is less Cl^- resorption and thus hyponatremia and hypochloremia may be present.
4. Concurrent acidemia associated with ketoacidosis stimulates the increased renal excretion of Cl^-, which would contribute to hypochloremia.

C. Osmotic factors need to be considered when there is a loss of H_2O due to osmosis in the intestine. Via osmosis alone, H_2O is lost, but Na^+ and Cl^- are not. However other factors affecting tbH_2O, $tbNa^+$, and total body Cl^- content may lead to normonatremic, normochloremic dehydration or hyponatremic, hypochloremic dehydration.

POTASSIUM (K^+) CONCENTRATION

I. Physiologic processes

A. Plasma [K^+] is nearly equivalent to ECF [K^+], which is mostly dependent on tbK^+ and movement of K^+ into and out of K^+-rich cells in response to changes in acid-base status. Therefore, plasma (or serum) [K^+] should be interpreted with consideration of acid-base status and potential variations in tbK^+ status.

B. Most cells are rich in K^+ because of a Na^+-K^+-ATPase pump that constantly pumps K^+ into cells against a concentration gradient. Na^+-K^+-ATPase helps maintain extracellular [K^+] relatively low so that certain cells (especially heart and skeletal muscle) can function. Acid-base factors alter the movement of K^+ in and out of cells; generally, acidemia promotes hyperkalemia whereas alkalemia promotes hypokalemia. Details are in respective hyperkalemia and hypokalemia sections.

C. If the acid-base status is normal, then serum or plasma [K^+] tends to reflect tbK^+, i.e., hyperkalemia tends to occur with increased tbK^+, whereas hypokalemia tends to occur with decreased tbK^+. However, a K^+-depleted state may occur before hypokalemia develops.[70]

D. Plasma [K^+] changes unrelated to acid-base status

1. K^+ distribution between ECF and ICF
   a. Epinephrine and insulin promote the uptake of K^+ into cells through the action of a Na^+-K^+-ATPase pump. This effect of insulin is independent of its actions on glucose uptake.
   b. Hyperkalemia promotes the cellular uptake of K^+, whereas hypokalemia promotes the loss of K^+ from cells.
2. Renal excretion of K^+
   a. Typically, K^+ is resorbed before the renal tubular fluid reaches the distal nephron. Therefore, the [K^+] in tubular fluid entering the distal nephron is near zero.
   b. K^+ is secreted primarily by the principal cells of the collecting ducts (Figure 9.2). Aldosterone promotes this process by stimulating Na^+-K^+-ATPase in the basolateral membrane and opening luminal K^+ channels. Hyperkalemia and angiotensin II are the major stimulants of aldosterone secretion. Adrenocorticotropic hormone (ACTH) and hyponatremia also stimulate aldosterone release, but the effect of hyponatremia is diminished if the effective blood volume is adequate.[1] Resorption of Na^+ without Cl^- (e.g., hypochloremic states) establishes an electrochemical gradient (tubular fluid more negative than cell) that promotes the secretion of K^+.
   c. A high flow rate of tubular fluid also promotes K^+ secretion because secreted K^+ is quickly washed away and thus does not inhibit the passive movement of K^+ from cells to tubular fluid. Conversely, a low flow rate inhibits K^+ secretion.
   d. ADH promotes K^+ channel activity and K^+ secretion in the cortical collecting ducts, thereby counterbalancing the reduced K^+ secretion that occurs with decreased urinary flow. The enhanced secretion prevents the hyperkalemia that might otherwise be caused by dehydration-induced oliguria.
   e. K^+ is conserved by type A intercalated cells of the cortical collecting ducts through H^+-K^+-ATPase pump activity when hypokalemia is caused by decreased tbK^+ (Figure 9.4).
3. Plasma [K^+] is also influenced by intestinal and cutaneous processes: absorption of dietary K^+ by the intestine, loss of K^+ via feces, and loss of K^+ via sweat.

II. Analytical concepts

A. Assays, terms, and units

1. Ion-selective electrode assays are the most common assays for measuring [K^+]. These assays measure the electrical potential of K^+ (potentiometry) and convert it to concentrations in plasma, serum, or whole blood. Flame photometers for measuring [K^+] were the analytical gold standard but are no longer used for routine patient sample analysis. K^+ is mostly a free ion in the plasma or serum H_2O.
2. Unit conversion: mEq/L × 1 = mmol/L, and mg/dL ÷ 3.9 = mmol/L (SI unit)[9]

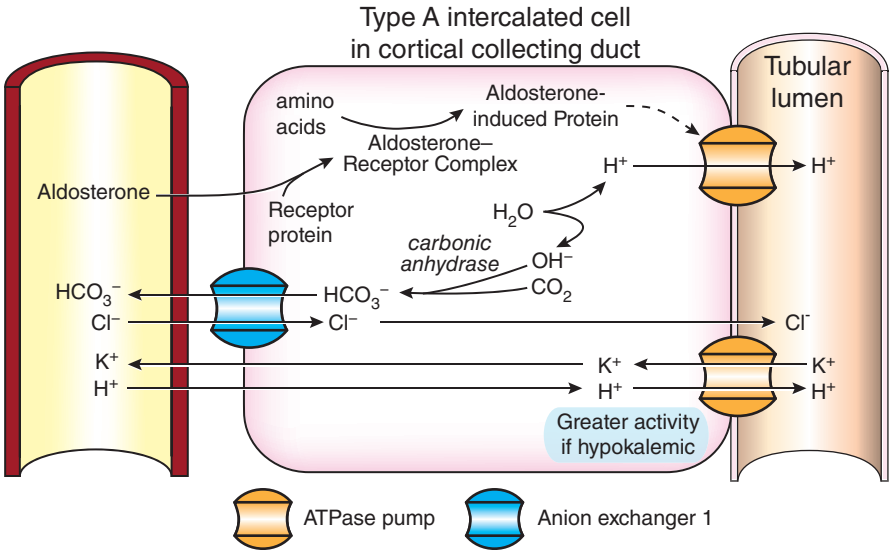


Figure 9.4. Secretion of H^+ by type A (Acid secreting) intercalated cells of the cortical collecting duct. Aldosterone enters the type A intercalated cells and binds to receptor proteins. The aldosterone-receptor complex stimulates the synthesis of aldosterone-induced proteins that may include components of the H^+-ATPase pump. Through the actions of the pump, which is more active during systemic acidemia, aldosterone causes the secretion of H^+ and formation of HCO_3^- through the following processes:

1. H^+ released from the dissociation of H_2O is pumped into the tubular lumen.
2. OH^- released from the dissociation of H_2O combines with CO_2 in the presence of carbonic anhydrase to form HCO_3^-, which is exchanged for Cl^- in the peritubular fluid.
3. Cl^- that enters the cell in the Cl^--HCO_3^- anion exchanger then enters the tubular lumen to maintain electroneutrality (balance the H^+ secretion).

The net result if stimulated by acidemia or aldosterone is increased H^+ and Cl^- secretion and increased HCO_3^- entering plasma. In an independent process, but in the same cells, a H^+-K^+-ATPase pump promotes the resorption of K^+ and secretion of H^+; this pump is more active when hypokalemia is present. Thus, hypokalemia promotes increased H^+ secretion.

B. Sample for $[K^+]$ quantitation
   1. Serum or heparinized plasma may be used. The $[K^+]$ in a serum or plasma sample is stable for months if the sample does not dehydrate, but false increases are expected with delayed separation of cells (see Pseudohyperkalemia). When feline whole blood samples were stored at about 20 °C for 20–28 h in clot or heparin tubes prior to centrifugation and analysis, $[K^+]$ increased from baseline by a median of 0.4 mmol/L for serum (up to 1.0 mmol/L) and 0.6 mmol/L for plasma (up to 1.5 mmol/L).[71] The K^+ in platelets, reticulocytes, and WBCs may all have contributed to the increases. Because platelet activation during clotting causes release of platelet K^+, RIs and patient results for plasma $[K^+]$ are mildly lower than those for serum $[K^+]$.
   2. K_3EDTA should not be used as an anticoagulant for testing plasma $[K^+]$ because the anticoagulant's K^+ will be added to the plasma and be measured. Similarly, K_3EDTA contamination of a sample during transfer of blood from a syringe to a K_3EDTA tube may cause subsequent transfer of K_3EDTA to a clot tube, thus falsely increasing $[K^+]$.

III. Hyperkalemia

A. This typically occurs when the renal excretion of K^+ decreases or K^+ shifts from ICF to ECF. Hyperkalemia may also occur with increased ingestion of K^+ or treatment with K^+, especially if there is renal compromise. If the acid-base status is normal, then serum $[K^+]$ tends to reflect tbK^+.

B. Disorders and pathogeneses (Table 9.5)

1. Shifting of K^+ from ICF to ECF (no change in tbK^+)

a. Acidemias of inorganic metabolic acidoses (Figure 9.5)[72]

(1) In metabolic acidosis, the increased $[H^+]$ and decreased $[HCO_3^-]$ reverse the physiologic actions that limit extracellular $[K^+]$ and instead promote an increase

Table 9.5. Disorders and conditions that cause hyperkalemia

Shifting of K^+ from ICF to ECF (no change in tbK^+)
Acidemias of inorganic metabolic acidoses (renal failure,* some diarrheas,* administration of NH_4Cl)[a]
Rhabdomyolysis or other muscle damage
Acquired: strenuous exercise, seizures, selenium deficiency, malignant hyperthermia
Congenital: dystrophin deficiency in cats, hyperkalemic periodic paralysis in quarter horses and perhaps dogs
Massive intravascular hemolysis in animals with K^+-rich RBCs
Other massive tissue necrosis
Hypertonicity shift
After exercise in hypothyroid dogs
Pseudohyperkalemia caused by in vitro hemolysis (species differences), release from platelets during clotting (with thrombocytosis), or release from leukemic cells
Increased tbK^+
Decreased renal excretion of K^+
Renal insufficiency or failure (primarily oliguric or anuric)*
Urinary tract obstruction or urine leakage into the body*
Hypoaldosteronism
Hypoadrenocorticism (Addison's)*
Angiotensin-converting enzyme inhibitors
Hyperreninemic hypoaldosteronism
Heparin-induced hyperkalemia
Trimethoprim-induced K^+ retention
Increased intake: administration of K^+-rich fluid
Other or unknown mechanism
Hyperkalemia associated with general anesthesia (rare)
Repeated drainage of chylous thoracic effusions
Peritoneal effusions in cats

* A relatively common disorder, condition, or mechanism

[a] Acidemias due to organic metabolic acidoses (e.g., lactic acidosis, ketoacidosis) and respiratory acidoses are not expected to cause hyperkalemia (see Potassium Concentration, Section III.B.1 and Figure 9.5).

in ECF [K^+]. The effects of acidemia and decreased [HCO_3^-] are explained in Figure 9.5.

(a) Acidemia due to disorders that cause metabolic inorganic acidoses (primarily caused by renal failure, but also by some diarrheas and administration of NH_4Cl) promotes hyperkalemia. The various types of inorganic acidoses – those associated with increases or decreases in molecules lacking carbon – are described in Chapter 10 (Acid-Base Abnormalities). For each 0.1 unit decrease in ECF pH, the plasma [K^+] was reported to increase 0.2–1.7 mmol/L.[1]

(b) In organic metabolic acidosis, the entry of H^+ and an A^- (e.g., L-lactate, AcAc, BHB) into cells results in cellular Na^+ loading which stimulates the Na^+-K^+-ATPase to cause K^+ entry into cells, thus promoting normokalemia (Figure 9.5D). Other factors also oppose the development of hyperkalemia.

(i) Kidneys excrete excess organic anions; accumulation of the organic anions in the tubular fluid obligates increased excretion of cations, including K^+.

(ii) An animal with a disorder causing an organic acidosis tends to have decreased intake of K^+ because of anorexia and vomiting.

(2) Respiratory acidoses are not associated with significantly altered serum or plasma [K^+] because of two differences from the metabolic states:

(a) In respiratory acidoses, the acidemia tends to reduce K^+ entry into cells but the increased [HCO_3^-] of compensatory metabolic alkalosis tends to shift K^+ into cells (Figure 9.5B and 9.5C).

(b) Non–acid-base factors that alter [K^+] in metabolic disorders, e.g., decreased renal K^+ excretion due to renal failure and increased H^+ production associated with lipolysis or hypoxic states, usually are not present in respiratory disorders.

b. Rhabdomyolysis or other muscle damage (release of K^+ from myocytes)

(1) Acquired disorders

(a) Strenuous exercise may cause hyperkalemia. Hyperkalemia is caused by K^+ release from active myocytes. The K^+ release might be related to ion shifts caused by a decrease in nondiffusible intracellular anions that occur as phosphocreatine, a highly dissociated acid, hydrolyzes to neutral creatine.[73] Also, myocytes may be damaged.

(b) Seizures resulting in muscle damage

(c) Selenium deficiency[61]

(d) Malignant hyperthermia[74]

(2) Congenital disorders: Rhabdomyolysis can be induced by stress or anesthetic in dystrophin-deficient cats,[75] and it occurs with hyperkalemic periodic paralysis in quarter horses[76] and possibly dogs (the dog's reported serum [K^+] was never > 5 mmol/L but did increase from 3.9 mmol/L to 4.9 mmol/L after exercise).[77]

c. Massive intravascular hemolysis in animals with K^+-rich RBCs: English springer spaniels with phosphofructokinase deficiency have hyperkalemia concurrent with their hemolytic episodes, which might involve K^+-rich young RBCs.[78] Hyperkalemia might occur with intravascular hemolysis in other animals that have K^+-rich RBCs (see Pseudohyperkalemia below).

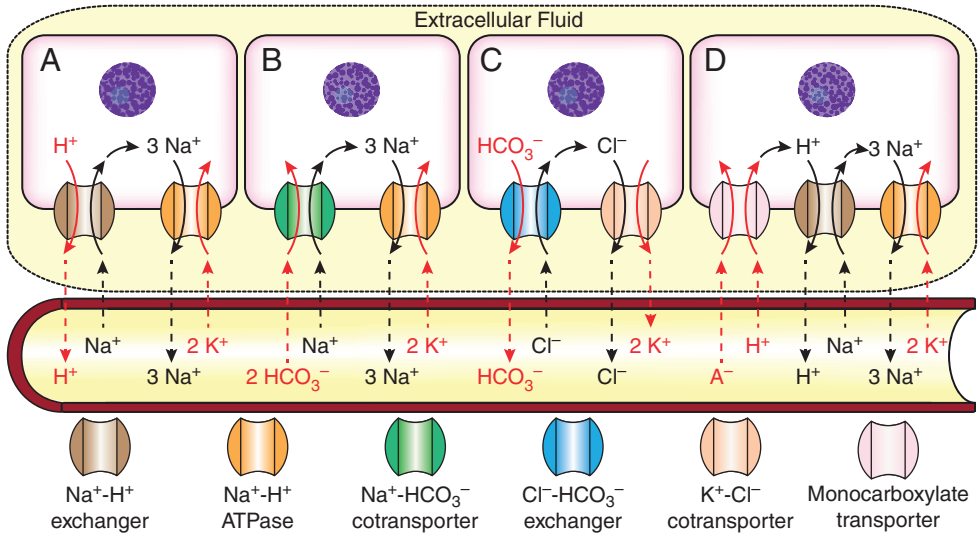


Figure 9.5. Multiple acid-base transport pathways affecting H^+ and K^+ shifts in metabolic acidosis with acidemia.[a,b]

In metabolic acidosis, the increased $[H^+]$ and decreased $[HCO_3^-]$ counteract the physiologic actions that shift K^+ from ECF to ICF and limit extracellular $[K^+]$. H^+ and K^+ do not simply exchange for each other.

- Cells A and B: Acidemia (*Cell A*) and decreased $[HCO_3^-]$ (*Cell B*) reduce the indirect exchange of Na^+ and K^+ by limiting cellular Na^+ uptake, making less Na^+ available to enable K^+ entry into cells, thereby contributing to hyperkalemia.
- Cell C: Decreased $[HCO_3^-]$ promotes HCO_3^--Cl^- exchange which promotes movement of K^+ to ECF via a K^+-Cl^- exchanger, thereby contributing to hyperkalemia.
- Cell D: In organic metabolic acidosis, the entry of H^+ and an A^- (e.g., L-lactate, AcAc, BHB) results in cellular Na^+ loading which stimulates the Na^+-K^+-ATPase to cause K^+ entry into cells; this counteracts the other effects of acidemia and decreased $[HCO_3^-]$, thereby promoting normokalemia.

Specific descriptions for each of cells A–D:

A. Na^+-K^+ exchange is the major pathway to pump K^+ from ECF to ICF; the process is promoted by Na^+-K^+-ATPase stimulation. When acidemia is present, the lower extracellular pH shifts the H^+ equilibrium to inhibit Na^+-H^+ exchange and thus H^+ remains in the ICF. The resulting decrease in intracellular Na^+ reduces Na^+-K^+-ATPase activity and decreases the active uptake of extracellular K^+, so ECF K^+ accumulates and contributes to hyperkalemia. Through this mechanism, acidemia promotes hyperkalemia by limiting the movement of K^+ into cells.

B. The Na^+-K^+-ATPase pump works together with a Na^+-HCO_3^- cotransporter. When there is a decreased ECF $[HCO_3^-]$ (metabolic acidosis), the lower ECF $[HCO_3^-]$ reduces the transport of Na^+ into the cell, which decreases the Na^+-K^+-ATPase pump activity. As above, this decreases the active uptake of extracellular K^+, and the accumulation of ECF K^+ contributes to hyperkalemia. Through this mechanism, decreased ECF $[HCO_3^-]$ promotes hyperkalemia by limiting cellular uptake of K^+. Because decreased $[HCO_3^-]$ in metabolic acidosis is accompanied by increased $[H^+]$, it appears as if there is a K^+-H^+ exchange, with the rise in $[H^+]$ increasing the ECF $[K^+]$ to cause hyperkalemia.

C. Cell membranes also have a Cl^--HCO_3^- exchanger that works in parallel with a K^+-Cl^- cotransporter. A decrease in the ECF $[HCO_3^-]$ (metabolic acidosis) causes more HCO_3^- to leave cells and more Cl^- to enter cells. The increase in ICF $[Cl^-]$ enhances the transport of K^+ from the cell, which contributes to the hyperkalemia. By this mechanism, decreased ECF $[HCO_3^-]$ promotes hyperkalemia by shifting K^+ out of cells. Again, the decreased $[HCO_3^-]$ is accompanied by an increased $[H^+]$ that makes it appear as if the rise in $[H^+]$ is causing hyperkalemia by increasing ECF $[K^+]$.

d. Other massive tissue necrosis: Massive tissue necrosis causing release of K^+-rich ICF from dead cells is also seen with tumor necrosis, arterial thrombosis with tissue ischemia, and sometimes just prior to death.

e. Hypertonicity shift: Hypertonicity (e.g., caused by hyperglycemia in diabetes mellitus) leads to an osmotic shift of H_2O from cells to ECF, thereby increasing the intracellular $[K^+]$ (but not total K^+ content). K^+ then shifts down a concentration gradient into ECF. Although the K^+ shift decreases the intracellular $[K^+]$, the net effect on the plasma $[K^+]$ depends on many other factors (e.g., renal loss of K^+ due to ketonuria and osmotic diuresis in diabetes mellitus), and thus hyperkalemia may not be present.

f. After exercise in hypothyroid dogs: Plasma $[K^+]$ increased, but only slightly exceeded the upper reference limit, when dogs with experimentally-induced hypothyroidism were exercised (running for 5 min); the increase did not occur in euthyroid dogs. The hyperkalemia may have been caused by an ICF to ECF shift of K^+ related to reduced Na^+-K^+-ATPase activity in skeletal muscle.[79] Hyperkalemia is not expected in nonexercised hypothyroid dogs, and hypothyroid dogs are not expected to be avid exercisers!

g. Pseudohyperkalemia

(1) In vitro hemolysis or leakage of K^+ because of delayed removal of serum or plasma from RBCs

(a) If the $[K^+]$ in RBCs is greater than the $[K^+]$ in plasma, then the loss of K^+ from RBCs may cause pseudohyperkalemia. The ratio of RBC $[K^+]$ to plasma $[K^+]$ varies among species and among breeds of dogs.

(b) This form of pseudohyperkalemia may occur in horses,[80] some dogs (i.e., Japanese Akitas,[81] shar peis,[82] Japanese shiba inus, and rarely others), many cattle (to a lesser extent), goats, some sheep,[11] and camels.[83] Serum or plasma may not appear hemolyzed despite substantial K^+ leakage from RBCs.

(c) As in most dogs, hemolysis does not cause hyperkalemia in cats, because RBC $[K^+]$ is similar to plasma $[K^+]$.

Figure 9.5. *continued*

D. In an organic acidosis with the accumulation of anions (A^-) such as L-lactate or BHB, H^+ and A^- enter cells. The higher ICF $[H^+]$ promotes more Na^+ entry into cells (Na^+ loading), which stimulates the Na^+-K^+-ATPase to cause K^+ to enter cells. Thus, hyperkalemia typically does not accompany an organic acidosis with acidemia.

[a] In Cells A, B, and C, the red lines indicate the initiating H^+ or HCO_3^- movement and the resulting K^+ movement. In Cell D, the red lines indicate the initial H^+ and A^- movements. In Cells A–D, the black lines show the movement of electrolytes that are coupled with the red-line actions. The solid lines primarily represent electrolyte movement through cell membrane channels. The dashed lines represent electrolyte diffusion between ECF pools (intravascular and extravascular).

[b] The electrolyte shifts associated with hypokalemia are not shown but basically would be opposite shifts. If there were hypokalemia due to K^+ depletion, the lower ECF $[K^+]$ would cause less K^+ entry and less exit of H^+ from Cell A, less K^+ and HCO_3^- entry into Cell B, and more K^+ and HCO_3^- exit from Cell C; the net effect would be toward metabolic alkalosis and alkalemia.

(2) Thrombocytosis[84]
   (a) K^+ is released from platelets when a blood sample clots. The released K^+ is part of the $[K^+]$ used to establish serum RIs. Without thrombocytosis, serum $[K^+]$ is about 0.3–0.5 mmol/L greater than plasma $[K^+]$ in feline samples collected from the same blood draw.[71,85] The difference between serum and plasma samples might be greater in some species if total platelet K^+ content is higher.
   (b) When thrombocytosis is marked (e.g., > 1,000,000/μL), more K^+ is added to the serum during clotting than normally occurs and thus the measured serum $[K^+]$ may be increased. Plasma $[K^+]$ would not be affected.
(3) Leakage of K^+ from leukemic cells when a neoplastic leukocytosis is present[86]
(4) Contamination of a sample with K_3EDTA-anticoagulated blood, which would also cause pseudohypocalcemia

2. Increased tbK^+
   a. Decreased renal excretion of K^+
      (1) Renal insufficiency or failure (primarily oliguric or anuric acute renal disease or the terminal stages of chronic renal disease)
         (a) In oliguric or anuric phases of acute or chronic renal failure, the rate of tubular fluid flow is low in the collecting ducts, and secreted K^+ accumulates in the tubular fluid; this reduces the K^+ concentration gradient between cells and tubular fluid. Because the movement of K^+ from cells is a passive process, K^+ secretion from the collecting duct cells is diminished. With this impairment of renal K^+ secretion, dietary K^+ or K^+ released from cells accumulates in plasma.
         (b) A reduction in renal blood flow reduces the amount of K^+ presented to the kidney for excretion and thus contributes to hyperkalemia.
         (c) The acidemia of renal failure and concurrent decreased $[HCO_3^-]$ contribute to hyperkalemia (Figure 9.5).
         (d) In chronic polyuric renal failure, the number of functional nephrons decreases, but the K^+ excretion per functioning nephron increases. Therefore, hyperkalemia is not expected in polyuric chronic renal failure.
      (2) Urinary tract obstruction or leakage
         (a) With urinary tract leakage (ruptured bladder or internal leakage from ureter or urethra), K^+ enters the ECF and thus is not removed from the body. With time, hyperkalemia may develop. In experimentally produced uroperitoneum, some dogs developed hyperkalemia within 2 d, and all dogs were hyperkalemic after 4 d.[63] Hyperkalemia is not always seen in spontaneous canine and feline cases of uroperitoneum, probably because of the variable durations and variable amounts of urine in the peritoneal cavities.
         (b) Urinary tract obstruction may similarly cause hyperkalemia because of the defective excretion of K^+ and the development of acidemia and decreased $[HCO_3^-]$ (Figure 9.5).
      (3) Hypoaldosteronism
         (a) Hypoadrenocorticism (Addison's disease)
            (i) Bilateral adrenocortical hypoplasia decreases the production of aldosterone and cortisol.

(ii) Hypoaldosteronemia decreases the activity of Na^+-K^+-ATPase pumps in the basolateral membranes of the principal cells of collecting ducts (Figure 9.2). This leads to decreased resorption of Na^+ and therefore decreased movement of K^+ into the tubular epithelial cells for passive secretion to the tubular lumen. This process is further inhibited because the number of luminal K^+ channels decreases with decreased aldosterone stimulus.

(b) Inhibitors of angiotensin-converting enzyme (ACE inhibitors) might cause hyperkalemia because of the development of hypoaldosteronism. However, if no underlying disease or concurrent drugs are impairing renal function, K^+ is secreted through aldosterone-independent processes, and thus only mild or no hyperkalemia occurs.[1]

(c) Selective hypoaldosteronemia without hypocortisolemia (hyperreninemic hypoaldosteronism): This rare condition was diagnosed in a 9-yr-old dog that had marked hyperkalemia, mild hyponatremia, mild hypochloremia, normocortisolemia, hypoaldosteronemia, and increased plasma renin activity.[87] The defect that led to the hypoaldosteronemia was not determined. Note: Increased renin activity is expected in hypoadrenocorticism in which there are reduced cortisol and aldosterone concentrations.

(d) Heparin-induced hyperkalemia:[88]

(i) An 11-yr-old dog with chronic renal failure was treated with continuous venovenous hemodiafiltration and concurrent constant-rate infusion of unfractionated heparin for anticoagulation. While receiving heparin, the dog developed hyperkalemia and concurrently had an [aldosterone] of 0 pmol/L. After cessation of heparin infusion, the normokalemia returned and baseline [aldosterone] was WRI.

(ii) Unfractionated and low-molecular-weight heparin have been shown to temporarily suppress aldosterone synthesis in people.[89]

(4) Trimethoprim-induced K^+ retention[90]

(a) Trimethoprim (especially when protonated in acidic urine) blocks the luminal Na^+ channels of the collecting ducts and thus acts like a K^+-sparing diuretic.[91,92] With reduced Na^+ resorption, the electrical gradient that promotes K^+ secretion is reduced.

(b) Effects of trimethoprim would be enhanced if the flow rate in the tubules were decreased, because K^+ secretion depends on a K^+ gradient maintained by high urine flow rates that carry secreted K^+ away, thus increasing the intracellular to lumen K^+ gradient.

b. Increased intake of K^+: administration of K^+-rich fluid,[93] especially if there is renal compromise

3. Other or unknown mechanisms

a. Rarely, unexplained hyperkalemia with associated clinical signs (e.g., bradycardia) has developed in dogs after at least 1 h of general anesthesia.[94]

b. Repeated drainage of chylous pleural effusions:[49,50] The pathogenesis of hyperkalemia is not established but may relate to concurrent hyponatremia. In the presence of hyponatremia and hypovolemia, much of the filtrate Na^+ is resorbed in the PCT and ascending LoH. If Na^+ is not delivered to the distal nephron, less Na^+ is resorbed distally and thus less K^+ secretion occurs.

c. Peritoneal effusions: Four cats with peritoneal effusions were hyperkalemic and either mildly or markedly hyponatremic.[95] The effusions were caused by abdominal carcinomatosis (one cat) and feline infectious peritonitis (two and possibly three cats; a firm diagnosis was not made in one cat). The pathogeneses of the hyponatremia and hyperkalemia were not established.

IV. Normokalemia in acidemic or alkalemic animals
  A. Normokalemia in an acidemic animal
    1. Because acidemia in disorders that cause inorganic metabolic acidosis is expected to increase serum [K^+], normokalemia in the presence of such an acidemia suggests an animal has a decreased tbK^+. Therapeutic correction of the acidemia may lower the serum [K^+] into the hypokalemic range. Disorders that may produce this pathophysiologic state include renal failure and some cases of diarrhea.
    2. As mentioned earlier (see Potassium Concentration, Section III.B.1.a(1)(b)), acidemic animals with an organic metabolic acidosis are not expected to be hyperkalemic because of ion shifts. This is because either the K^+ shift into cells is accompanied by an organic anion shift, or renal excretion of K^+ is increased to maintain electroneutrality as organic anions are excreted.
  B. Normokalemia in an alkalemic animal
    1. This combination of findings is not expected because disorders that cause a metabolic alkalosis are expected to mildly decrease serum [K^+].
    2. If normokalemia were detected in the presence of alkalemia, it would suggest an increased tbK^+, and therapeutic correction of the alkalemia might raise the serum [K^+] into the hyperkalemic range.

V. Hypokalemia
  A. This typically occurs when there is a shift of K^+ from ECF to ICF, a decreased dietary intake of K^+, or an increased K^+ loss via kidneys, alimentary tract, or skin. If the acid-base status is normal, then the serum [K^+] tends to reflect tbK^+.
  B. Disorders and pathogeneses (Table 9.6)
    1. Shifting of K^+ from ECF to ICF (no change in tbK^+)
      a. Metabolic alkalosis with alkalemia may cause hypokalemia via multiple mechanisms. The decrease in plasma [K^+] was reported to be 0.4 mmol/L for each 0.1 unit increase in ECF pH.[1]
        (1) When there is an increased plasma [HCO_3^-], it enhances Na^+ entry into cells. The greater ICF [Na^+] stimulates the Na^+-K^+-ATPase pump which moves K^+ into cells, thus reducing ECF [K^+] and contributing to hypokalemia (Figure 9.5B). Also, the greater ECF [HCO_3^-] reduces HCO_3^--Cl^- exchange; less ICF Cl^- reduces the transport of K^+ out of cells, thus contributing to a reduced plasma [K^+] (Figure 9.5C).
        (2) Increased plasma [HCO_3^-] leads to excessive HCO_3^- in renal tubular fluid which obligates the renal loss of cations, including K^+.
        (3) When metabolic alkalosis is due to vomiting or abomasal dysfunction, decreased dietary intake of K^+ contributes to hypokalemia.
        (4) Lastly, animals with metabolic alkalosis tend to be hypovolemic and thus an activated RAAS leads to increased renal K^+ excretion.
      b. Insulin surge: Insulin promotes the cellular uptake of K^+, probably through the activation of a Na^+-K^+-ATPase. Administration of exogenous insulin, or a sudden

Table 9.6. Disorders and conditions that cause hypokalemia
Shifting of K^+ from ECF to ICF (no change in tbK^+)
Metabolic alkalosis with alkalemia
Insulin surge (insulin injection, IV glucose, xylitol ingestion)
Endotoxemia
β_2-adrenergic stimulation, endogenous (shock, fear) or exogenous (salbutamol, salmetrol)
Methylxanthine toxicity (chocolate, caffeine)
Barium toxicosis (soluble forms)
Decreased tbK^+
Decreased K^+ intake: anorexia or other reasons for not eating*
Increased excretion of K^+
Increased renal loss
Increased fluid flow in nephron:* osmotic diuresis, Na^+-losing nephropathies, therapeutic diuresis (including loop diuretics, thiazide, and acetazolamide)
Increased renal excretion of anions (ketonuria, lactaturia, bicarbonaturia)*
Vomiting or sequestration of H^+ and Cl^- causing hypochloremic metabolic alkalosis*
Hyperaldosteronism, primary
Bovine hypokalemia syndrome
Periodic hypokalemic polymyopathy in Burmese cats
Increased alimentary loss:* vomiting, diarrhea, sequestration, or excess salivation
Increased cutaneous loss: sweating in horses
Hypokalemic renal failure in cats*

* A relatively common disorder, condition, or mechanism

release of endogenous insulin after IV glucose administration or xylitol ingestion (dogs and cats), may cause hypokalemia. A ketotic diabetic animal, in contrast to a nonketotic one, would more likely develop hypokalemia because it might have decreased tbK^+ (see Potassium Concentration, Section V.B.2.b(1)(b)).

c. Experimental endotoxemia causes hypokalemia.[96,97] The hypokalemia may develop because endotoxin stimulates the Na^+-K^+-ATPase of skeletal muscle, which causes K^+ to shift from the ECF to muscle fibers.[96] The endotoxin-induced release of insulin may also contribute to the K^+ shift.

d. β_2-adrenergic stimulation (see Lactate Concentration, Section III.C.2): Catecholamines released endogenously in states of fear, shock, or other critical illness can stimulate myocyte Na^+-K^+-ATPase and cause K^+ to shift from the ECF to myocytes.[98] Exogenous β_2-adrenergic agonists (salbutamol, salmetrol) act similarly.[99]

e. Methylxanthine toxicity (chocolate, caffeine, theobromine, theophylline)[100] (see Lactate Concentration, Section III.C.2)

f. Barium (Ba^{2+}) toxicosis:[101] If an animal ingests a soluble form of barium (e.g., barium carbonate in a rodenticide or barium nitrate from a sparkler), barium is absorbed and competitively blocks cell membrane channels through which K^+ leaves cells. Because K^+ continues to enter cells via Na^+-K^+-ATPase pumps, there is a net shift of K^+ from plasma to cells.[102,103] Hypokalemia can be marked.

2. Decreased tbK^+
   a. Decreased K^+ intake
      (1) Because most diets are K^+ rich, anorexia or prolonged food restriction can contribute to decreased tbK^+. However, hypokalemia was not present in starved dogs.[104]
      (2) The severity of hypokalemia will be enhanced if other processes lead to K^+ loss or shifting to ICF. For example, hypokalemia in llamas and alpacas with gastrointestinal disease is reportedly worse with greater severity and duration of anorexia.[105]
   b. Increased excretion of K^+
      (1) Increased renal loss
         (a) Increased tubular flow
            (i) Increased flow of fluid through the collecting ducts allows for increased secretion of K^+ from the tubular cells. Rapid flushing of K^+ maintains a low tubular [K^+], which allows the passive movement of K^+ out of the cells.
            (ii) Polyuric states that promote hypokalemia include glucosuria, Na^+-losing nephropathies (pyelonephritis, tubular interstitial nephritis, and possibly hypercalcemic nephropathy), and diuretic use (LoH diuretics, thiazide, and acetazolamide).
         (b) Increased renal excretion of anions
            (i) Ketonuria: AcAc and BHB (ketone bodies) are anions that are in the tubules during ketosis. Their negative charges add to the electrochemical gradient that promotes K^+ excretion, especially when there is concurrent stimulation of Na^+ resorption. Animals with ketoacidotic diabetes mellitus tend to be hypokalemic; however, the net K^+ balance is the result of multiple concurrent processes.
            (ii) Lactaturia: Lactate is poorly resorbed by renal tubules. Thus, increased renal excretion of L-lactate with hyperlactatemia obligates the loss of cations such as Na^+ and K^+.
            (iii) Bicarbonaturia: When plasma [HCO_3^-] exceeds the renal capacity to conserve HCO_3^- (renal threshold) or if there is a proximal tubular acidosis, the HCO_3^- that remains in the tubular fluid obligates the renal excretion of cations such as Na^+ and K^+.
         (c) Vomiting or sequestration of H^+ and Cl^- that causes hypochloremic metabolic alkalosis (e.g., left or right displaced abomasum, bovine intussusception, bovine hemorrhagic bowel syndrome, or other disorders causing upper gastrointestinal obstruction)
            (i) During the development of this metabolic alkalosis, plasma [HCO_3^-] may increase enough that the tubular maximum for HCO_3^- is exceeded and thus HCO_3^- is presented to the distal nephron. Because HCO_3^- is not resorbed in the distal nephron, its presence adds to the electrochemical gradient that promotes tubular K^+ secretion.
            (ii) Renal secretion of K^+ (and concurrently H^+) is promoted through the actions of aldosterone if an animal is hypovolemic.
            (iii) Some K^+ in gastric secretions may be lost via vomiting. Also, animals with these disorders typically are not eating well and thus have decreased dietary intake of K^+.

(d) Hyperaldosteronism, primary

(i) Hypokalemia is a major diagnostic feature of primary hyperaldosteronism in cats[106–108] and in dogs.[19,109,110] Adrenal neoplasms (carcinomas and adenomas) are the major causes of hyperaldosteronism; it may also be caused by idiopathic adrenal hyperplasia[106,111] or renin-secreting neoplasms.[112] Note: In contrast to renin-secreting neoplasms, low plasma renin activity is expected with the autonomous production of aldosterone in primary hyperaldosteronism.

(ii) Increased aldosterone concentrations in these conditions promote renal K^+ secretion by stimulating Na^+-K^+-ATPase and opening K^+ channels in the collecting ducts (Figure 9.2).

(iii) Other than hypokalemia, the major presenting features are hypertension, increased [aldosterone], and presence of an adrenal neoplasm or hyperplasia. Other common abnormalities include increased [HCO_3^-] or [tCO_2] (metabolic alkalosis) (due to aldosterone activity and hypokalemia), and increased creatine kinase activity (due to hypokalemic myopathy). The persistent hypertension can produce sufficient renal damage to cause renal failure.[111]

(iv) Normonatremia is expected due to "aldosterone escape," but a slight hypernatremia can be found (see III. Hypernatremia, B.2.b.).

(v) Confirmatory testing is presented in Chapter 18.

(e) Bovine hypokalemia syndrome: This term has been applied to lactating and younger dairy cattle with hypokalemia accompanied by weakness that may progress to recumbency and rhabdomyolysis. Multiple pathogeneses may be involved. The combination of anorexia and repeated systemic or intramammary doses of isoflupredone acetate, a glucocorticoid, contribute to the development of hypokalemia in some cattle; the mineralocorticoid activity of isoflupredone acetate results in increased renal excretion of K^+.[113] Other reported cases of the hypokalemia syndrome involve dexamethasone administration or treatments with dextrose and insulin.

(f) Periodic hypokalemic polymyopathy in Burmese cats

(i) This hereditary disorder is a homozygous, recessive trait caused by a nonsense mutation in *WNK4*.[114] This gene codes for an enzyme (lysine-deficient 4 protein kinase, a "with no lysine kinase") involved in Na^+/K^+ exchange in the distal nephron. The defect most likely results in a K^+-wasting nephropathy.[115]

(ii) The Burmese kittens or cats have hypokalemia, muscle weakness, and high serum creatine kinase activities. The myopathy is precipitated by stress or exercise.

(2) Increased alimentary loss of K^+: vomiting, diarrhea, sequestration, or excess salivation

(a) Vomiting may cause loss of K^+-rich fluid and reduced intestinal absorption of K^+ and thus lead to hypokalemia. Also, enhanced renal excretion of K^+ (a response to increased aldosterone activity and decreased availability of Cl^-) may add to a decreased tbK^+. See Section V.B.2.b(1) above.

(b) With diarrheas, loss of electrolytes and H_2O may be massive. H_2O intake may contribute a dilutional component to the hypokalemia. In addition,

lack of absorption of dietary K^+ may augment the severity of the tbK^+ depletion and thus hypokalemia.

(c) K^+-rich fluid may be sequestered in the intestines of animals with ileus, especially in horses.

(d) Because saliva is a K^+-rich fluid, loss of large volumes of saliva (because of choking, a lacerated salivary gland, dysphagia, or ptyalism) in horses and cattle can produce hypokalemia.[116] Initially, the concurrent loss of HCO_3^- might produce a mild acidosis. If the animals also become hyponatremic, hypochloremic, and hypovolemic, then renal compensations (conservation of Na^+ and HCO_3^-, and excretion of K^+ and H^+) may lead to a hypokalemic metabolic alkalosis.

(3) Increased cutaneous loss: sweating in horses

(a) Equine sweat is relatively K^+ rich as compared to plasma. Thus, profuse sweating can lead to K^+ loss, tbK^+ depletion, and hypokalemia.[32–35]

(b) If the horse is drinking, H_2O intake would promote dilution of the remaining K^+.

c. Hypokalemic renal failure in cats

(1) Cats with progressive renal diseases that cause chronic renal failure are prone to tbK^+ depletion (*kaliopenia*) and thus development of hypokalemia.

(2) The exact pathogenesis of the hypokalemia of chronic renal failure is unknown and may be due to multiple factors: increased renal K^+ excretion, increased colonic K^+ excretion, and decreased dietary K^+ intake. Possibly, because cats retain renal concentrating ability much longer in renal disease than do other domestic mammals, their collecting ducts may remain responsive to aldosterone during hypovolemia and thus retain Na^+ and secrete K^+. In experimental conditions, a NaCl-restricted diet did result in hypokalemia and inappropriate kaliuresis when the glomerular filtration rate was reduced.[117]

SODIUM TO POTASSIUM (Na^+:K^+) RATIO

I. Calculating a Na^+:K^+ ratio may enhance detection of electrolyte disorders, as it may be outside the RI when $[Na^+]$ and $[K^+]$ are still WRI.

A. The Na^+:K^+ ratio is obtained by dividing a measured serum or plasma $[Na^+]$ by a measured serum or plasma $[K^+]$ (both expressions in mmol/L or mEq/L).

B. A low Na^+:K^+ ratio is common and expected in hypoadrenocorticism. It has been written that a Na^+:K^+ ratio < 27 (or 25 or 22 or 20…) is diagnostic of hypoadrenocorticism in dogs. The lowest ratios are found when hyponatremia and hyperkalemia are concurrent, but a low ratio is not unique to hypoadrenocorticism.

II. Causes of a decreased Na^+:K^+ ratio:[118,119] A decreased Na^+:K^+ ratio is not required to detect the following disorders, but it suggests possible abnormalities in Na^+ or K^+ concentrations.

A. Hypoadrenocorticism (Addison's disease)

1. Hyponatremia is caused by increased renal excretion of Na^+ (due to decreased aldosterone) and retention of H_2O (due to increased ADH secondary to hypocortisolemia).

2. Hyperkalemia is caused by decreased renal secretion of K^+ (due to decreased aldosterone).

B. Diarrhea (dogs, cats, and horses)
   1. Hyponatremia is caused by increased intestinal loss of Na^+, perhaps accompanied by increased H_2O intake. Horses with severe diarrhea are more prone to low Na^+:K^+ ratios than are dogs and cats.
   2. Hyperkalemia may occur because of acidemia associated with HCO_3^- loss.
   3. When seen with a whipworm (*Trichuris vulpis*) infection, the disorder has been called pseudo-Addison's disease. Neither adrenocortical hypoplasia nor hypoaldosteronism are present, and responsive hyperaldosteronism is expected.

C. Renal failure (dogs and cats)
   1. Hyponatremia is caused by a decreased ability of tubules to resorb Na^+.
   2. Hyperkalemia is caused by a decreased ability to secrete K^+ (primarily in oliguric states).

D. Urinary tract obstruction (dogs and cats) or uroperitoneum
   1. Mild hyponatremia might be found during obstruction. If uroperitoneum is present, plasma Na^+ may diffuse into Na^+-poor peritoneal fluid to create hyponatremia.
   2. Hyperkalemia caused by obstruction occurs primarily because of acute oliguria or anuria leading to decreased tubular secretion of K^+. Uroperitoneum may cause hyperkalemia via K^+ diffusion from the peritoneal cavity to blood. Acidemia may contribute to hyperkalemia in both types of hyperkalemia.

E. Diabetes mellitus with ketonuria (dogs and cats)
   1. Hyponatremia is caused by increased renal Na^+ excretion (due to osmotic diuresis and obligated cation excretion) and plasma dilution (a shift of H_2O from ICF to ECF because of marked hyperglycemia).
   2. Hyperkalemia is not expected with ketoacidosis. It is much more common for [K^+] to be WRI or decreased.

F. Third-space loss (pleural or peritoneal effusions) (dogs and cats)[50,95]
   1. Hyponatremia may result from the dilution of ECF [Na^+] by the H_2O that is retained through hypovolemic ADH and thirst responses. Drainage of effusions, e.g., repeated drainage of chylous effusions, would remove ECF Na^+ and thus enhance the severity of the hyponatremia.
   2. Hyperkalemia may result from one or more processes, including decreased renal excretion of K^+ (caused by decreased distal tubular flow rate or hyponatremia) or a shift from ICF to ECF because of associated acidemia.

G. Others
   1. Any disorder that causes hyponatremia without a corresponding decrease in serum [K^+] will lower the Na^+:K^+ ratio.
   2. Any disorder that causes hyperkalemia without a corresponding increase in serum [Na^+] will lower the Na^+:K^+ ratio.

CHLORIDE (Cl^-) CONCENTRATION

I. Physiologic processes
   A. Plasma [Cl^-] is nearly equivalent to ECF [Cl^-], which is influenced by ECF concentrations of Na^+ and HCO_3^-. Therefore, complete interpretation of plasma or serum [Cl^-] requires knowledge of [Na^+] and at least a consideration of an animal's acid-base status.
   B. Control of plasma [Cl^-]
      1. Renal Cl^- resorption and secretion[1]
         a. About 75 % of filtrate Cl^- is resorbed in the PCTs down a concentration gradient created by Na^+ and H_2O resorption and through a formate-Cl^- exchanger (formate is the conjugate base of formic acid and an intermediate in one-carbon metabolism). Angiotensin II stimulates resorption of Na^+, Cl^-, and H_2O in the PCTs.

b. Cl^- is actively resorbed in the thick ascending limb of the LoH via a Na^+-K^+-$2Cl^-$ transporter in the luminal membrane. The rate-limiting factor is Cl^- delivery to the LoH. This process is blocked by loop diuretics (e.g., furosemide) and is stimulated by ADH.
c. Cl^- is passively resorbed in the distal nephron by an electrochemical gradient established by Na^+ movement through Na^+ channels. Aldosterone promotes Na^+ resorption in the collecting ducts.
d. Na^+ and Cl^- resorption in the distal nephron also involves an aldosterone-independent Na^+-Cl^- cotransporter. This process varies directly with Na^+ delivery to the DCT. Thiazide diuretics block this cotransporter.
e. Type A intercalated cells of the collecting ducts secrete Cl^- when H^+ is secreted. The process is stimulated by acidemia (Figure 9.4). Conversely, when there is an alkalemia or HCO_3^- excess, Cl^- is conserved and HCO_3^- is secreted by type B intercalated cells (Figure 9.9).

2. Alimentary tract functions pertaining to Cl^-
a. Stomach: Gastric (or abomasal) mucosa secretes HCl as part of the digestive process. Secretion of Cl^- requires the generation of HCO_3^- which increases plasma [HCO_3^-] (Figure 9.6).

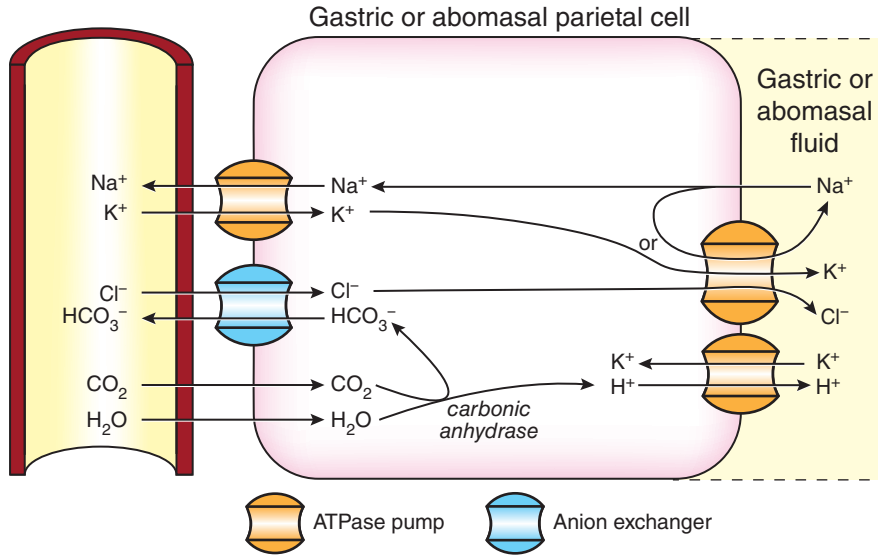


Figure 9.6. Gastric or abomasal secretion of H^+ and Cl^-.
H^+ and HCO_3^- are formed in parietal cells from the combination of OH^- (from H_2O) and CO_2 in a reaction catalyzed by carbonic anhydrase. H^+ (from H_2O) is secreted into the lumen via a H^+-K^+-ATPase pump. The generated HCO_3^- is transported to the ECF via a Cl^--HCO_3^- exchanger. Cl^- is actively pumped into the lumen with Na^+ or K^+, but most Na^+ and K^+ ions are resorbed, thus leaving H^+ and Cl^- in the lumen. Basolateral Na^+-K^+-ATPase pumps promote favorable electrochemical gradients for K^+ secretion and subsequent recycling through H^+-K^+-ATPase pumps, and for Na^+ resorption from the gastric or abomasal lumen. The major results of the process are gastric secretion of H^+ and Cl^-, a lesser plasma [Cl^-], and a greater plasma [HCO_3^-].

b. Small intestine: In healthy animals, Cl^- that has passed into the intestinal tract can be absorbed electroneutrally via apical Cl^--HCO_3^- exchangers (HCO_3^- secreted) that function in parallel with apical Na^+-H^+ exchangers (Na^+ absorbed, H^+ secreted).[120] This is the major route of absorption. It is inhibited with stimulation of Cl^- secretion through cystic fibrosis transmembrane conductance regulator (CFTR) channels; this is accompanied by paracellular Na^+ excretion. (Note: CFTR channels are the abnormal Cl^- channels in cystic fibrosis.)

c. Colon: Cl^- absorption involves Cl^--HCO_3^- exchange that may or may not be Na^+ dependent.[121] HCO_3^- secretion also involves Cl^- independent methods (see Bicarbonate and Total Carbon Dioxide Concentrations).

II. Analytical concepts

A. Terms and units

1. Assays measure the electrical potential of Cl^- (potentiometry) or [Cl^-] (spectrophotometry or coulometric/amperometric titration) in plasma, serum, or whole blood. The Cl^- is mostly a free ion in the plasma or serum H_2O.
2. Unit conversion: mEq/L × 1 = mmol/L, and mg/dL ÷ 3.55 = mmol/L (SI unit)[9]

B. Sample for [Cl^-] quantitation

1. Serum or heparinized plasma may be used. [Cl^-] is stable for months if the sample does not dehydrate.
2. Excess heparin from underfilling tubes or overheparinizing syringes may react with some ion-selective electrodes to produce a positive interference of 2–10 mmol/L.[122]

C. Principles of assays

1. Ion-selective electrode assays are the most common, but there are different types of electrodes. Those that contain an ion-exchanger (e.g., a quaternary ammonium chloride) may detect any anion with a hydration energy greater than that of Cl^-; thus, there may be positive interference from bromide, iodide, thiocyanate, thiosulfate, salicylate, HCO_3^-, nitrate, and heparin.[123] Solid-state silver/silver chloride electrodes are not affected by the organic anions, but positive interference can occur with barium, iodide, and thiosulfate.[124] Substantial interference often reflects a toxicity (e.g., salicylate, nitrate, bromide) or treatment (e.g., bromide, thiosulfate, nitrate), but changes in endogenous substances can be significant.
   a. Bromide may be present when potassium bromide is used as an anticonvulsant or when excessive bromide is ingested.[125] False increases can be marked and cause falsely negative AGs.
   b. A marked reduction in [HCO_3^-] can produce a falsely low [Cl^-],[126] and a marked increase in [HCO_3^-] can cause a falsely high [Cl^-].[127] This is because the measured [Cl^-] by these assays includes a HCO_3^- contribution; the interference can be reduced by modifying the pH of reagents.[128]
   c. Other anions (e.g., lactate and BHB) may react to give mildly increased values; the Cl^- value may be increased about 3 mmol/L by lactate at 10 mmol/L and by BHB at 16 mmol/L (i-STAT literature).
2. If measurement of [Cl^-] in ruminal or forestomach fluid is needed, then a chloridometer should be used. Ion-selective electrode methods give falsely high concentrations because other anions, which may be present in various and unknown quantities, react with the Cl^- electrode.[129]

3. When measured with the i-STAT, Cl^- concentrations were falsely increased in 56 % of people with azotemia, and the interference was replicated by urea-spiked samples.[130] The [Cl^-] was falsely increased by about 2–14 mmol/L when [UN] was > 56 mg/dL.
4. Coulometric/amperometric titration assays have mostly been replaced by direct or indirect potentiometry using ion-selective electrodes.

III. Hyperchloremia
   A. This typically occurs when there is hypernatremia but occasionally is found concurrently with a decreased [HCO_3^-] (i.e., hyperchloremic metabolic acidoses). Conceptually, changes in [Cl^-] are related to attempts to maintain electroneutrality. As [Na^+] increases, so does [Cl^-]. As [HCO_3^-] decreases, [Cl^-] increases.
   B. Disorders and pathogeneses (Table 9.7)

Table 9.7. Disorders and conditions that cause hyperchloremia

H_2O-deficit group (hyperchloremic and hypernatremic disorders)
Inadequate H_2O intake
H_2O deprivation*
Defective thirst response (hypothalamic defect)
Solute-poor H_2O loss (without adequate H_2O replacement)
Insensible loss:* panting, hyperventilation, or fever
Diabetes insipidus (central or nephrogenic)
Adrenocortical neoplasm secreting corticosterone and aldosterone
H_2O loss > Na^+ and Cl^- loss
Renal: osmotic diuresis
Alimentary: osmotic diarrhea, osmotic sequestration, PO_4 enemas, ruminal acidosis, or paintball toxicosis
Cl^--excess group (increased total body Cl^- content)
Excess Cl^- with concurrently restricted H_2O intake
Salt poisoning
Administration of hypertonic saline, KCl, or NH_4Cl
Decreased renal excretion of Na^+ and Cl^- (hyperaldosteronism)
Reset or defective osmoreceptor (essential hypernatremia)
Hyperchloremic metabolic acidosis
Alimentary loss of HCO_3^-*
Renal loss of HCO_3^-
Proximal renal tubular acidosis (inherited or acquired)
Distal renal tubular acidosis
Respiratory alkalosis (chronic)
Other or unknown mechanisms
Sample dehydration or sublimation (spurious hyperchloremia)
Pseudohyperchloremia: bromide interference, marked hypoproteinemia, markedly increased [HCO_3^-], excessive heparin, rarely other anions (interference depends on method)

* A relatively common disorder, condition, or mechanism

1. H_2O-deficit group (hyperchloremic and hypernatremic disorders): Because Cl^- is the major anion that helps maintain electroneutrality in the ECF, disorders that cause hypernatremic dehydration also tend to create hyperchloremia (see Sodium Concentration, Section III.B.1).
2. Cl^--excess group (increased total body Cl^- content) (usually with concurrent Na^+ excess)
   a. Excess Cl^- with concurrently restricted H_2O intake
      (1) Salt poisoning
      (2) Administration of hypertonic saline, KCl, or NH_4Cl
   b. Decreased renal excretion of Na^+ and Cl^- (hyperaldosteronism) (rare)
      (1) Excessive aldosterone promotes excessive renal Cl^- (and Na^+) retention.
      (2) Hyperchloremia (and hypernatremia) may occur if H_2O is restricted or ADH activity is reduced. Aldosterone escape may prevent hyperchloremia (see Sodium Concentration, Section III.B.2b).
   c. Reset or defective osmoreceptor (essential hypernatremia) (see Sodium Concentration, Section III.B.2c)
3. Hyperchloremic metabolic acidosis
   a. Alimentary loss of HCO_3^-
      (1) Vomiting or diarrhea that causes a loss of HCO_3^--rich intestinal secretions may lead to HCO_3^- depletion and hyperchloremic metabolic acidosis.
      (2) Cattle that do not ingest saliva (because of an esophageal obstruction) become HCO_3^- depleted because their saliva is rich in HCO_3^-.
      (3) For most parts of the intestines, the HCO_3^- secretions are also Na^+ rich but Cl^- poor because the HCO_3^- secretion is linked to Cl^- absorption; the enhanced Cl^- absorption leads to hyperchloremia.
   b. Renal loss of HCO_3^- (see Bicarbonate and Total Carbon Dioxide Concentrations, Section IV.C)
      (1) With proximal renal tubular acidosis, the reduced reclamation of HCO_3^- may allow more Cl^- to be resorbed with Na^+, and thus hyperchloremic metabolic acidosis may develop.
      (2) With distal renal tubular acidosis, the impaired ability to secrete H^+ might be linked to the failure of a Cl^--HCO_3^- shuttle, thus impairing secretion of Cl^- and reclamation of HCO_3^-.
4. Respiratory alkalosis (chronic)
   a. As a compensatory change to prolonged hypocapnia and alkalemia, the renal retention of H^+ is increased and thus the renal conservation of HCO_3^- is reduced.
   b. This compensatory fall in plasma [HCO_3^-] is balanced by an increase in [Cl^-] and concentrations of other unidentified anions.[131]
5. Other or unknown mechanisms
   a. Sample dehydration or sublimation: Exposure of serum or plasma to air may allow evaporation that causes spurious hyperchloremia. This is especially true when air-conditioning systems blow cool dry air over the sample processing or analysis areas. Sublimation of H_2O from frozen samples may cause apparent hyperchloremia.
   b. Pseudohyperchloremia: As noted in the analytical section, the presence of bromide or other anions may result in falsely increased chloride concentrations. If [Cl^-] is measured by indirect potentiometry, then marked hypoproteinemia would create a mild false increase in the [Cl^-] (see pseudohypernatremia above). Pseudohyperchloremia may also occur when there is a markedly increased [HCO_3^-] and when the [Cl^-] is determined by common quaternary ammonium ion-selective electrode assays (see Chloride Concentration, Section II.C.1c).

IV. Normochloremia: Recognizing the presence of normochloremia may be important when either hypernatremia or decreased serum [HCO_3^-] is present, because it suggests the presence of an increased AG (see the Anion Gap section).

V. Hypochloremia
   A. This typically occurs when there is (i) hyponatremia (and pseudohypochloremia when there is pseudohyponatremia), (ii) an increased serum [HCO_3^-] as occurs with metabolic alkaloses, or (iii) an increased AG. It may also occur with metabolic acidosis because Cl^- excretion accompanies the enhanced renal excretion of H^+ via NH_4^+ excretion (Figure 9.7) or via type A intercalated cells (Figure 9.4).
   B. Disorders and pathogeneses (Table 9.8)

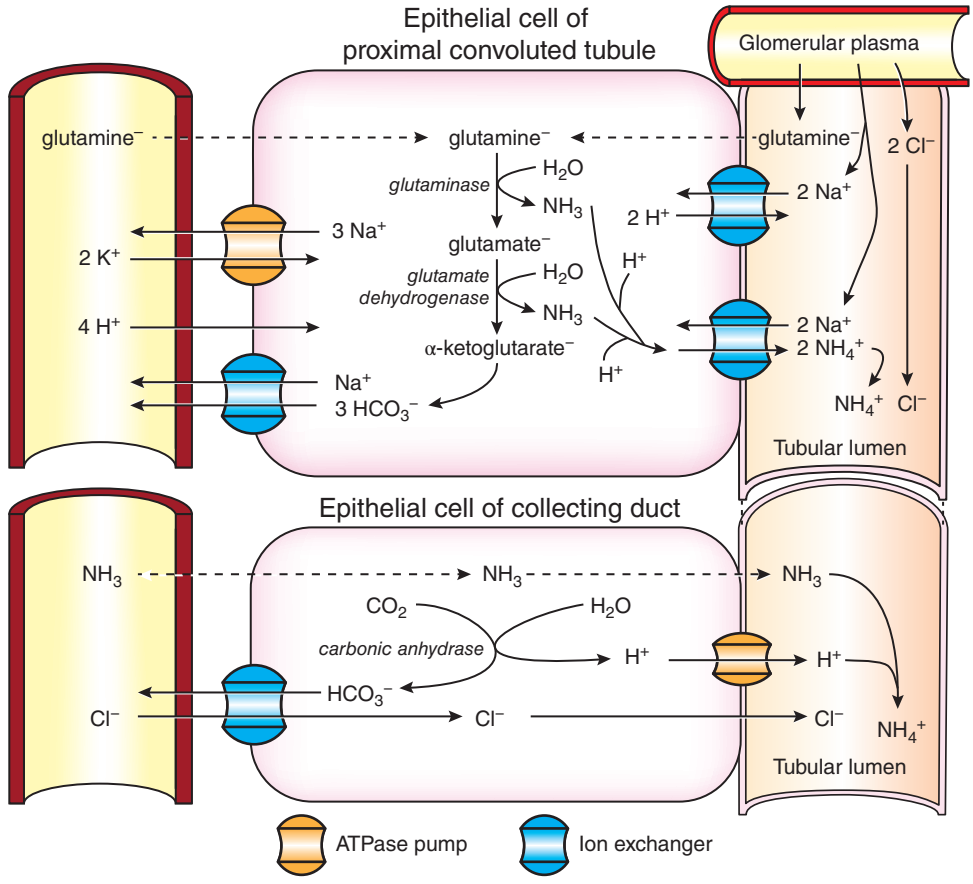


Figure 9.7. Renal excretion of H^+ via NH_4^+.[132]

Epithelial cell of proximal tubule

- Glutamine, an amino acid, is the major source of renal NH_4^+ production. It passes freely through the glomerular filtration barrier and is almost entirely resorbed in the proximal tubules. With acidemia, myocyte glutamine synthetase activity increases, resulting in increased arterial, filtrate, and proximal tubular cell glutamine.

1. Cl^--deficit group (net Cl^- loss > H_2O loss): There are two major groups of hypochloremic disorders, those with concurrent hyponatremia and those without concurrent hyponatremia. Hypochloremia in the absence of hyponatremia suggests the presence of metabolic alkalosis (with an increased [HCO_3^-]) or metabolic acidosis with an increased AG (with an increased concentration of an anion other than Cl^- or HCO_3^-) because electroneutrality must be maintained. Interpretation of HCO_3^- (or tCO_2) and

Figure 9.7. *continued*

- Acidemia (also hypokalemia or high dietary protein) promotes renal ammoniagenesis and excretion by increasing proximal tubular cell expression of genes for (i) glutamine transporters on basolateral and apical membranes (within minutes) and (ii) enzymes for glutamine degradation (over days). It also decreases the opposing basal activity of glutamine synthetase, so less glutamate combines with NH_4^+ to form glutamine (not shown); undegraded glutamine is transported back to blood. The increased uptake of glutamine from plasma and tubular fluid and degradation of glutamine by glutaminase and glutamate dehydrogenase in mitochondria releases NH_3 which combines with H^+ (ultimately from plasma) to form NH_4^+ (unclear if before or after transport from mitochondria).
- In health, about 50 % of the generated NH_4^+ returns to blood via basolateral transport (not shown). A lower percentage returns to blood with acidemia because more of the NH_4^+ is secreted into the tubular lumen in exchange for Na^+. This most likely occurs primarily by NH_4^+ substituting for H^+ in the Na^+-H^+ exchanger, but parallel NH_3 and H^+ secretion may occur. This increased renal excretion of NH_4^+ obligates the excretion of anions (primarily Cl^-, as it is the major anion in proximal tubular fluid) to maintain electroneutrality.
- The α-ketoglutarate (from glutamine degradation) enters the Krebs cycle and is oxidized in the mitochondria, producing HCO_3^-. The Krebs cycle also generates malate, which is transported from mitochondria and used for gluconeogenesis, a process that also generates HCO_3^-. The HCO_3^- is transported out of the cell via a Na^+-$3HCO_3^-$ exchanger, thus increasing plasma HCO_3^-, which buffers H^+. The apical Na^+-H^+ exchanger promotes conservation of Na^+ and excretion of H^+. Energy for electrolyte transport is provided by the $3Na^+$-$2K^+$-ATPase pump (creates gradients).
- In summary, the severity of acidemia is reduced by increases in (i) H^+ combining with NH_3 to form NH_4^+, (ii) excretion of H^+ in NH_4^+, (iii) direct excretion of H^+, and (iv) the generation of HCO_3^- which can buffer ECF H^+. Net HCO_3^- formation is proportional to the amount of NH_4^+ that is excreted, not formed, because NH_4^+ that returns to plasma enters hepatocytes to form urea or glutamine in reactions that consume HCO_3^-.

Loop of Henle (not shown): NH_3 secretion continues in the descending limb of the LoH followed by NH_4^+ resorption in the thick ascending limb. An interstitial concentration gradient is created with [NH_4^+] increasing from cortex to inner medulla.

Epithelial cell of collecting duct

- In the collecting duct, it is likely that both NH_3 and NH_4^+ move from ECF to cells via Rhesus transporters, but some NH_3 may diffuse from the ECF into cells and then the tubular lumen. Most of the NH_3, including that formed from dissociation of NH_4^+, appears to reach the tubular lumen through Rhesus transporters in parallel with H^+ secretion via H^+-ATPase (and likely H^+-K^+-ATPase) pumps. Interstitial NH_4^+ also enters cells of the inner medullary collecting ducts by substituting for K^+ in basolateral Na^+-K^+-ATPase pumps, particularly when hypokalemia is present (not shown).
- Acidemia stimulates the activity of H^+-ATPase pumps, thus promoting excretion of H^+ formed by the carbonic anhydrase reaction. The luminal H^+ binds NH_3 to form NH_4^+, thus reducing luminal [NH_3] and enhancing the movement of NH_3 from plasma through epithelial cells and into the tubular fluid to promote more NH_4^+ formation and therefore H^+ secretion.
- The HCO_3^- formed by the carbonic anhydrase reaction can exchange with plasma Cl^- via a HCO_3^--Cl^- exchanger, and the Cl^- may diffuse into the tubular fluid through apical Cl^- channels, maintaining electroneutrality.
- This renal compensation for acidemia increases the renal excretion of H^+ (in NH_4^+), generates HCO_3^-, and may increase the renal excretion of Cl^-, thus producing a compensatory hypochloremic metabolic alkalosis.

Table 9.8. Disorders and conditions that cause hypochloremia
Cl^--deficit group: net Cl^- loss > H_2O loss (loss of Cl^--containing fluid followed by increased H_2O intake)
Concurrent loss of Na^+
Alimentary loss:* vomiting, diarrhea, sequestration, canine whipworm infection, excess salivation, bovine hemorrhagic bowel syndrome
Renal loss:* hypoadrenocorticism, prolonged diuresis, thiazide diuretics, Na^+-wasting nephropathies, hyperreninemic hypoaldosteronism
Cutaneous loss: sweating in horses
Third-space loss: repeated drainage of chylous effusion, acute hemorrhage or exudation
Metabolic alkaloses
Loss or sequestration of H^+ and Cl^-:* vomiting, left or right displaced abomasum, pyloric obstruction, bovine hemorrhagic bowel syndrome, camelid forestomach or intestinal obstruction
Bovine renal failure*
Loop diuretic (e.g., furosemide, torasemide) toxicosis
Thiazide diuretics
Metabolic acidoses with increased AGs
Ketoacidosis*
Lactic acidosis*
Ingestion of foreign substance (e.g., ethylene glycol) that generates anions
Compensatory renal metabolic alkalosis
H_2O-excess groups (with or without edema)
Edematous disorders
Congestive heart failure*
Hepatic cirrhosis
Nephrotic syndrome
Expanded ECF volume (but without edema)
Syndrome of inappropriate ADH secretion
Excess administration of Cl^--poor fluids
Primary polydipsia
Shifting of H_2O from ICF to ECF
Hyperglycemia*
Mannitol infusion (IV)
Shifting of Cl^- from intravascular to extravascular fluid
Uroperitoneum
Pseudohypochloremia
Hyperlipidemia, hyperproteinemia
Markedly decreased [HCO_3^-]

* A relatively common disorder, condition, or mechanism

AG values should be sufficient to identify these conditions, but calculations for Na^+-adjusted [Cl^-] and SID may be used. See the following section (VI) for Na^+-adjusted (Corrected) Chloride Concentration and the section on Strong-Ion Difference and Stewart's Method of Acid-Base Analysis in Chapter 10.

a. Concurrent loss of Na^+: Because Cl^- is the major anion that helps maintain electroneutrality in the ECF, disorders that cause hyponatremic dehydration via alimentary, urinary, cutaneous, or third-space loss tend to also create hypochloremia (see Sodium Concentration, Section V.B.1, and Table 9.4).
b. Metabolic alkaloses
   (1) Loss or sequestration of H^+ and Cl^- (e.g., vomiting, displaced abomasum, other upper gastrointestinal tract obstruction, gastric reflux in horses, bovine hemorrhagic bowel syndrome, or forestomach or intestinal obstruction in camelids)[133,134]: Loss of Cl^--rich secretions leads to depletion of Cl^- in the ECF and thus hypochloremia.
   (2) Bovine renal failure: Cattle with renal insufficiency tend to become alkalotic. The acid-base changes may result from abomasal atony, which creates a functional obstruction and thus sequestration of Cl^- (see Bicarbonate Concentration, Section III.B.1). Also, cattle excrete more K^+ via saliva during renal failure. When swallowed, the K^+ may limit the absorption of Cl^-.[135]
   (3) Loop diuretic toxicosis: Loop diuretics (e.g., furosemide, torasemide) compete for the Cl^--binding site in the Na^+-K^+-$2Cl^-$ transporter of the luminal membrane of the thick ascending limb of the LoH and thus inhibit Cl^- resorption. Excess excretion of Cl^- (relative to H_2O) causes hypochloremia. Concurrent hypokalemic metabolic alkalosis may occur (see Bicarbonate Concentration, Section III.B.1b).
   (4) Thiazide diuretics act in the DCT by interfering with Cl^- resorption via a Na^+-Cl^- cotransporter. Because this process occurs in the cortical nephron, it does not diminish medullary hypertonicity and thus the kidneys maintain concentrating ability. Thus, when stimulated by ADH, H_2O resorption causes hyponatremia and hypochloremia by dilution.
c. Metabolic acidoses with increased AGs
   (1) Ketoacidosis and lactic acidosis: In ketoacidosis and lactic acidosis, there is an increased renal clearance of poorly resorbable anions (ketone bodies and L-lactate, respectively) from plasma. These anions obligate the renal excretion of Na^+ to maintain electroneutrality. Thus, less Na^+ is resorbed in the PCTs and collecting ducts. Because Cl^- resorption in those tubules depends on the electrochemical gradient established by Na^+ resorption, the diminished Na^+ resorption diminishes the Cl^- resorption.[1]
   (2) Ingestion of a foreign substance that generates anions (e.g., ethylene glycol): Pathogenesis of the hypochloremia parallels that found with ketoacidosis and lactic acidosis, because renal excretion of poorly resorbable anions obligates Na^+ loss. Because Cl^- resorption frequently depends on Na^+ resorption, Na^+ loss leads to Cl^- loss. Other examples of exogenous anionic substances are listed in the Anion Gap section.
d. Compensatory renal metabolic alkalosis: If there is adequate renal function and there is not a renal tubular acidosis, the renal response to acidemia is to increase the excretion of H^+ by the following processes that increase the excretion of H^+ and Cl^- and the production of HCO_3^-. Thus, hypochloremia is an expected finding when kidneys are compensating for an acidemia. The net effect is to reduce the severity of the acidemia (excretion of H^+ and generation of HCO_3^- for buffering) and reduce plasma $[Cl^-]$.

(1) Acidemia stimulates the type A intercalated cells (Figure 9.4) to secrete H^+ and Cl^- and produce HCO_3^-.
(2) Acidemia stimulates the increased renal excretion of NH_4^+ in both the PCT and DCT epithelial cells (Figure 9.7); the increased excretion of NH_4^+ obligates excretion of anions such as Cl^-. However, this mechanism of acid (and Cl^-) excretion in the tubules appeared to be impaired when dogs were fasted for 2 d; decreased NH_4^+ excretion might help maintain muscle mass during fasting.[136]

2. H_2O-excess group (see Sodium Concentration, Section V.B.2): edematous disorders or expanded ECF volume without edema
3. Shifting of H_2O from ICF to ECF (see Sodium Concentration, Section V.B.3): hyperglycemia, IV mannitol infusion
4. Shifting of Cl^- from intravascular to extravascular space (see Sodium Concentration, Section V.B.5): uroperitoneum
5. Pseudohypochloremia
   a. The same factors (hyperlipidemia and hyperproteinemia) that cause pseudohyponatremia also cause pseudohypochloremia if the $[Cl^-]$ is measured by indirect potentiometry or coulometric/amperometric titration assays (see Sodium Concentration, Section V.B.7).
   b. Pseudohypochloremia may occur when there is a markedly decreased $[HCO_3^-]$ if the $[Cl^-]$ is determined by common ion-selective electrode assays (see Chloride Concentration, Section II.C.1c).

VI. Sodium-adjusted (Corrected) Chloride Concentration

A. As described in previous sections, changes in the tbH_2O status of an animal can lead to concurrent hypernatremia and hyperchloremia (e.g., dehydration with solute-poor H_2O loss) or concurrent hyponatremia and hypochloremia (e.g., H_2O retention in edematous states or the syndrome of inappropriate ADH secretion). When the altered concentrations are due to changes in tbH_2O status because of hemoconcentration or hemodilution, changes in $[Na^+]$ are expected to be matched by proportional changes in $[Cl^-]$. For example, a 10 % increase in $[Na^+]$ (e.g., from 140 to 154 mmol/L) should be accompanied by a 10 % increase in $[Cl^-]$ (e.g., from 100 to 110 mmol/L). If percentage $[Cl^-]$ changes do not match percentage $[Na^+]$ changes, it suggests that factors other than tbH_2O status are affecting the $[Cl^-]$.

B. This concept can be expressed as a ratio because a change in $[Na^+]$ should be matched by a proportionate change in $[Cl^-]$, so the ratios of the expected values in health to the values in disease should be the same for Na^+ and Cl^- (Eq. 9.3a). Using the example concentrations just given in A, it is seen that the ratio of $[Cl^-]$ before and during dehydration is equal to the $[Na^+]$ ratio before and during dehydration (Eq. 9.3b). Rearrangement of Eq. 9.3a yields Eq. 9.3c. Using that format, a Na^+-adjusted $[Cl^-]$ is calculated with a patient's $[Na^+]$ and $[Cl^-]$ and an appropriate $[Na^+]$ RI (Eq. 9.3d). As the patient's $[Na^+]$ is usually not known prior to an illness, a midpoint value of the $[Na^+]$ RI for a given laboratory is used for the $[Na^+]_{healthy}$ value.

$$\frac{[Cl^-]_{healthy}}{[Cl^-]_{diseased}} = \frac{[Na^+]_{healthy}}{[Na^+]_{diseased}} \quad (\mathbf{9.3a})$$

$$\frac{100\ mmol/L}{110\ mmol/L} = 0.91 = \frac{140\ mmol/L}{154\ mmol/L} \quad (\mathbf{9.3b})$$

$$[Cl^-]_{healthy} = [Cl^-]_{diseased} \times \frac{[Na^+]_{healthy}}{[Na^+]_{diseased}} \quad (\mathbf{9.3c})$$

Because the $[Na^+]$ for the patient before the current illness is typically not known, a midpoint value of the $[Na^+]$ RI is used:

$$Na^+\text{-adjusted } [Cl^-] = [Cl^-]_{patient} \times \frac{[Na^+]_{midpoint\ of\ RI}}{[Na^+]_{patient}} \quad (\mathbf{9.3d})$$

C. The Na^+-adjusted $[Cl^-]$ value has been called the $[Cl^-]_{corrected}$. However, using the "corrected" expression implies that the patient's measured $[Cl^-]$ was erroneous even though it represented the patient's true $[Cl^-]$.

D. If the Na^+-adjusted $[Cl^-]$ is higher or lower than the limits of the $[Cl^-]$ RI, factors not directly linked to tbH_2O status should be considered. For example:
   1. Hypochloremia may be due to renal excretion of Cl^- (without Na^+ loss), as seen with a renal response to acidemia (Figure 9.7) or with furosemide use for congestive cardiac failure.
   2. Hypochloremia may be due to increased gastrointestinal loss of Cl^- (without Na^+ loss), which occurs because the gastric mucosa produces HCO_3^- prior to secreting H^+ (Figure 9.6).
   3. Movement of Na^+ and Cl^- can be based on electrical neutrality, e.g., tubular resorption of one Na^+ ion is linked to resorption of one Cl^- ion. This one-to-one process occurs in the aldosterone-independent Na^+-Cl^- cotransporter of the DCTs and is blocked by thiazide diuretics. In this situation, Cl^- loss relative to Na^+ loss is one-to-one (not proportional) and thus greater than the ratio related to tbH_2O status.

E. The Na^+-adjusted $[Cl^-]$ calculation is based on assumptions and estimations and thus should be interpreted cautiously (loosely) because of biological and analytical variations.
   1. A major assumption is that all animals start with Na^+ and Cl^- concentrations at the midpoints of their respective RIs. However, many individuals have values nearer the reference limits, and an individual with a relatively low or high $[Na^+]$ does not necessarily have a relatively low or high $[Cl^-]$. This assumption of the midpoint concentration therefore yields additive errors and substantial imprecision. Based on analysis of a reference population of 70 dogs, Na^+-adjusted $[Cl^-]$ would better be interpreted in comparison to the Cl^- RI (not the midway value), because the adjusted values ranged from the lower to the upper Cl^- reference limit (authors' unpublished data).
      a. The RI and midpoint for $[Cl^-]$ were 106–115 mmol/L and 110 mmol/L, respectively. The mean $[Cl^-]$ was 112 mmol/L. The RI and midpoint for $[Na^+]$ were 139–150 mmol/L and 144 mmol/L, respectively. The mean $[Na^+]$ was 146 mmol/L. The Na^+-adjusted $[Cl^-]$ values for these 70 healthy dogs were 106–117 mmol/L, very similar to the $[Cl^-]$ RI.
      b. Of the 70 healthy dogs, 11 (16 %) had a Na^+-adjusted $[Cl^-]$ more than 3 mmol/L different from the midpoint of the $[Cl^-]$ RI, and therefore may have been considered by some to have a mismatch. However, only two had Na^+-adjusted $[Cl^-]$ values outside the $[Cl^-]$ RI. These findings support using the $[Cl^-]$ RI rather than its midpoint to decide if there is evidence that factors other than tbH_2O status are affecting the $[Cl^-]$.

2. Frequently, there is an assumption that the RI or RI's midpoint concentration for one laboratory also applies to another laboratory's data even when it is known that there is not complete analytical agreement in Na^+ and Cl^- laboratory methods. If the equation is used, then a RI or midpoint concentration appropriate for the laboratory method (the same as used for patient's $[Na^+]$ and for establishing current RIs) should be used.
3. When interpreting a Na^+-adjusted $[Cl^-]$, it should be remembered that multiple processes may concurrently affect $[Cl^-]$. For example, a dehydrated, vomiting dog might or might not have a decreased Na^+-adjusted $[Cl^-]$, depending on the net effect of multiple factors such as increased retention of Na^+ and Cl^- due to hypovolemia (more Na^+ than Cl^- retained), increased Cl^- loss due to vomiting (more Cl^- loss than Na^+ loss), and decreased H_2O intake because of vomiting (proportional changes in Na^+ and Cl^-). As with most laboratory data, a measured or calculated value should be interpreted with a thorough knowledge of the animal's clinical signs and current pathologic states.
4. The analytical precision of Na^+ and Cl^- assays typically is very good (i.e., repeated analysis of the same sample yields the same concentration ± 2 mmol/L), but this degree of imprecision could affect the calculated value and its interpretation.

F. A Na^+-adjusted $[Cl^-]$ typically yields the same conclusions as a more subjective evaluation ("eyeballing") of electrolyte concentrations.
1. If the $[Na^+]$ is only slightly decreased and the $[Cl^-]$ is moderately to markedly decreased, then there must be a pathologic process that is altering $[Cl^-]$ independently of the altered $[Na^+]$. The Na^+-adjusted $[Cl^-]$ would be decreased.
2. If there is an increased AG (see later section), there is an increased concentration of an anion other than Cl^- or HCO_3^-. The hypochloremia is associated with (not caused by) the accumulation of those anions and not entirely due to an altered $[Na^+]$. The Na^+-adjusted $[Cl^-]$ would be decreased.
3. If there is a decreased $[Cl^-]$ and an increased $[HCO_3^-]$ (i.e., hypochloremic metabolic alkalosis), then the hypochloremia is associated with a Cl^--HCO_3^- shuttle and not due to altered $[Na^+]$. The Na^+-adjusted $[Cl^-]$ would be decreased. Note: A Cl^--HCO_3^- shuttle is a common process for transferring Cl^- and HCO_3^- between ICF and ECF (Figures 9.4, 9.6, 9.7, and 9.9) and it concurrently maintains electroneutrality.
4. The increased $[Cl^-]$ of a hyperchloremic metabolic acidosis is also associated with a Cl^--HCO_3^- shuttle and not due to altered $[Na^+]$. The Na^+-adjusted $[Cl^-]$ would be increased.

BICARBONATE (HCO_3^-) AND TOTAL CARBON DIOXIDE (tCO_2) CONCENTRATIONS

I. Physiologic processes
A. HCO_3^- is a major buffer that helps maintain blood pH at physiologic concentrations. HCO_3^- is produced from H_2O and CO_2 in cells that have carbonic anhydrase, including RBCs, cells of the PCT (luminal and intracellular), parietal cells of the gastric and abomasal epithelium, intercalated cells (type A and B) of the collecting ducts, and exocrine pancreatic epithelial cells.
B. Plasma $[HCO_3^-]$ is dependent on its relationship with H^+ and dissolved $CO_{2(g)}$ as expressed by the Henderson–Hasselbalch equation (Eq. 9.4). If the pCO_2 remains constant and $[H^+]$ changes, then $[HCO_3^-]$ must change. If $[H^+]$ remains constant and the pCO_2 changes, then $[HCO_3^-]$ must change. Additional aspects of these relationships are explored in Chapter 10.

$$pH = 6.1 + \log \frac{[HCO_3^-]}{[H_2CO_3]}; \quad [H_2CO_3] = pCO_2 \times 0.0307 \qquad (9.4)$$

C. In gastric or abomasal mucosal cells, secretion of H^+ and Cl^- is accomplished by the utilization of Cl^- from the ECF and by the generation of HCO_3^- (Figure 9.6).
D. As described in the Cl^- section, parallel actions of a Cl^--HCO_3^- exchanger and a Na^+-H^+ exchanger result in HCO_3^- secretion in the small intestine.
E. In the colon, HCO_3^- secretion occurs by three processes:[121,137]
   1. The major process was thought for many years to be Cl^--HCO_3^- exchange. It is still considered an important process but probably not the major process.
   2. The major process appears to be an exchange of HCO_3^- and short-chain fatty acids such as butyrate, propionate, and acetate.
   3. There also is a process by which HCO_3^- is secreted via cystic fibrosis transmembrane conductance regulator channels.
F. In the PCTs, 90 % of filtrate HCO_3^- is conserved (not directly resorbed as HCO_3^-) (Figure 9.8). This occurs during H^+ secretion that is dependent on Na^+ resorption and mediated by the Na^+-H^+ antiporter.

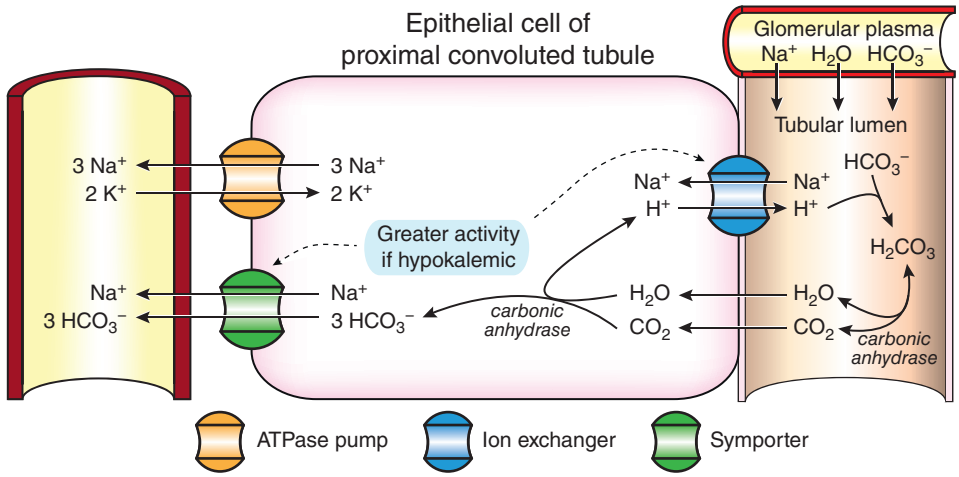
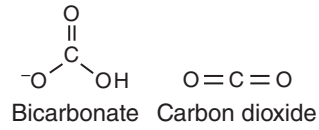


Figure 9.8. Conservation of HCO_3^- in PCTs.
H^+ secreted by a luminal border Na^+-H^+ exchanger combines with filtrate HCO_3^- to form H_2CO_3. Dissociation of H_2CO_3 into CO_2 and H_2O is facilitated by luminal carbonic anhydrase. After passive absorption of CO_2 and H_2O, cellular carbonic anhydrase helps CO_2 and OH^- (from H_2O) combine to form HCO_3^-, which is transported to the peritubular fluid via a Na^+-$3HCO_3^-$ symporter (cotransporter). The H^+ released from the H_2O is available for secretion in the next cycle. The Na^+-K^+-ATPase in the basolateral membrane creates the Na^+ gradient that promotes the Na^+-H^+ exchange in the luminal membrane. The electrolyte movement in the figure is not balanced; movement of other electrolytes (K^+, Cl^-, and NH_4^+) are involved to maintain electroneutrality. The net result of the process is HCO_3^- conservation and Na^+ resorption, but the HCO_3^- that enters the peritubular fluid is not the same HCO_3^- molecule that entered the filtrate. Thus HCO_3^- is not directly resorbed (HCO_3^- is a not a resorbable anion). The luminal Na^+-H^+ exchanger and the basolateral Na^+-$3HCO_3^-$ symporter are more active during hypokalemia, thus promoting greater H^+ secretion and HCO_3^- conservation.

G. Collecting ducts
1. HCO_3^- produced by type A (Acid secreting) intercalated cells of the collecting ducts enters the peritubular fluid through a Cl^--HCO_3^- exchanger. This process is linked to H^+ secretion by H^+-ATPase and is promoted by aldosterone (Figure 9.4).
2. HCO_3^- can be secreted (when there is a metabolic alkalosis) through a Cl^--HCO_3^- exchanger in type B (Base secreting) intercalated cells of the collecting ducts (Figure 9.9).

II. Analytical concepts
A. Terms and units



1. The $[HCO_3^-]$ is calculated during blood gas analysis of heparinized blood after measuring the blood pH and pCO_2 (Eq. 10.1a).
2. The $[HCO_3^-]$ in serum or plasma can be measured by clinical chemistry assays (see Principles of assays below).
3. The $[HCO_3^-]$ in serum or plasma can be estimated indirectly by measuring the $[tCO_2]$. tCO_2 reflects the total amount of CO_2 gas that can be liberated from serum. At a physiologic pH of 7.4, about 95 % of the potential CO_2 gas is in the form of HCO_3^-, ~ 5 % is in dissolved CO_2, and < 1 % is in carbamino compounds. Therefore, the $[tCO_2]$ is nearly equal to the $[HCO_3^-]$ (Eq. 10.3).
4. Unit conversion: mEq/L × 1 = mmol/L (SI unit)[9]

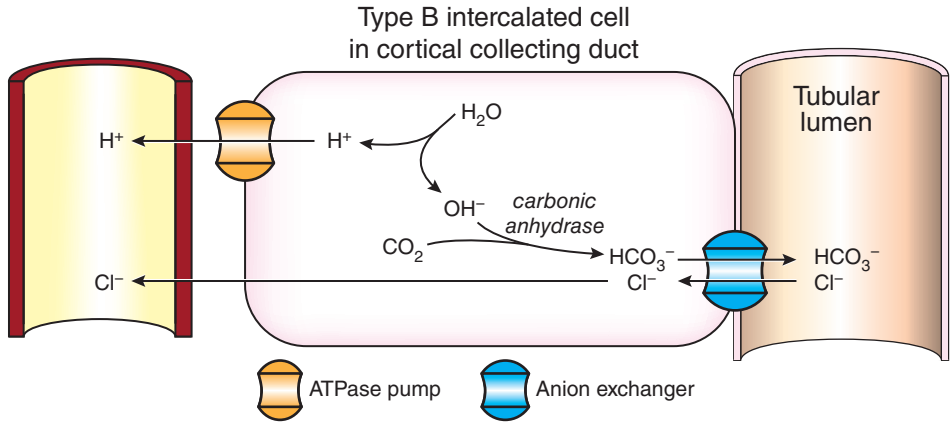


Figure 9.9. Secretion of HCO_3^- by type B (Base secreting) intercalated cells of the cortical collecting duct. When there is a metabolic alkalosis with increased plasma $[HCO_3^-]$ and alkalemia, type B intercalated cells can secrete HCO_3^- into the tubular lumen and concurrently generate H^+. With respect to HCO_3^- and H^+, type B intercalated cells essentially have the opposite function of type A intercalated cells. The net result is increased renal excretion of HCO_3^- and increased H^+ and Cl^- in plasma.

B. Sample for [HCO_3^-] measurement
   1. Serum or plasma may be used.
   2. Samples should have minimal exposure to air (a few minutes is acceptable). Because the pCO_2 of air is near 0 mmHg, $CO_{2(g)}$ diffuses from the sample to air. The lowering of the pCO_2 in the sample shifts the equilibrium toward CO_2 and away from HCO_3^-, thus lowering the [HCO_3^-] and [H^+] of the sample (Eq. 9.5). The same reaction occurs in vivo during expiration (as shown in Figure 10.1) to remove H^+ from blood, but the decrease in [HCO_3^-] is not detected because there is relatively much more HCO_3^- than H^+ (mmol/L vs nmol/L, respectively) and the HCO_3^- that is used is replaced.

$$H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow H_2O + CO_2 \rightarrow CO_2 \text{ lost to air} \qquad (\mathbf{9.5})$$

   3. Serum [tCO_2] in underfilled blood collection tubes is less than in filled tubes after contact with RBCs for about 20 min; it is about 2 mmol/L less for 3 mL of blood in a 10 mL tube, and almost 4 mmol/L less for 1 mL in a 10 mL tube.[138] RBCs may contribute to loss of CO_2 into underfilled tubes' dead spaces (which initially would be devoid of CO_2), because carbonic anhydrase in RBCs accelerates the conversion of HCO_3^- to CO_2. Even greater loss of CO_2 occurs with exposure to air before or after centrifugation.
   4. The size of the collection tube also affects the loss of CO_2 from the sample when blood is collected to the appropriate volume for each size of tube. The greatest loss occurs with the smaller collection tubes because there is a greater air-to-blood volume ratio in the smaller tubes after blood is collected.[139] In the described study, the mean plasma [tCO_2] in filled 10-mL tubes was near 30.5 mmol/L whereas the mean [tCO_2] in filled 2-mL tubes was near 29 mmol/L (plasma obtained within 30 min of blood collection).
   5. Additional information regarding sample collection and handling are in Chapter 10.

C. Principles of assays (examples provided)
   1. Vitros enzymatic tCO_2 assay and HCO_3^- assays of Hitachi and Olympus analyzers
      a. In a very alkaline environment, nearly all CO_2 is in the form of HCO_3^-. HCO_3^- reacts with phosphoenolpyruvate to form oxaloacetate, which is coupled to a reaction that consumes NADH. The consumption of NADH is proportional to [tCO_2] or [HCO_3^-], and is measured via spectrophotometry.
      b. Positive interference (most often noted in horses with rhabdomyolysis)
         (1) LD activity of 4500 U/L increases the [tCO_2] by 3 mmol/L because it promotes consumption of NADH as it catalyzes the conversion of serum L-lactate to pyruvate (Vitros method package insert). Oxamate is incorporated into some assays to inhibit LD and reduce, but not necessarily prevent, the interference.
         (2) Extreme increases in serum LD activity can result in substantial false increases in [HCO_3^-], e.g., > 40 mmol/L more than the actual concentration.[140]
   2. Beckman analyzer: In a colorimetric enzymatic assay for "CO_2, bicarbonate," sample HCO_3^- and phosphoenolpyruvate are converted to oxaloacetate and $H_2PO_4^-$ in a reaction catalyzed by phosphoenolpyruvate carboxylase. Malate dehydrogenase then catalyzes the reduction of oxaloacetate to form malate and NAD^+, the latter causing a measurable decrease in absorption proportional to the [HCO_3^-]. Consumption of HCO_3^- in the first reaction promotes conversion of dissolved CO_2 to HCO_3^-, which is measured, so results have been considered equivalent to [tCO_2].[141]

III. Increased bicarbonate (HCO_3^-) or total carbon dioxide (tCO_2) concentrations
   A. Increased [HCO_3^-] indicates the presence of a metabolic alkalosis. It may be (i) primary, (ii) renal compensation for a respiratory acidosis, or (iii) renal compensation for nonrenal causes of metabolic acidosis. Metabolic alkaloses are typically produced by the generation of HCO_3^- by gastric parietal cells or renal tubular cells. As [HCO_3^-] increases and if there is not a corresponding increase in the pCO_2, the blood pH increases (i.e., shifts toward alkalemia) (Eq. 9.4).
   B. Disorders and pathogeneses (Table 9.9)
      1. Production of HCO_3^- coupled with loss of H^+ from the body
         a. Gastric production: vomiting or pyloric obstruction (functional or mechanical)
            (1) Secretion of H^+ by the gastric mucosa generates HCO_3^- (Figure 9.6). If the H^+ is lost by vomiting, drainage of stomach contents (horses), or sequestration proximal to the intestine, it is not resorbed in the intestine. Therefore, the generated HCO_3^- is not used to buffer the H^+ that would otherwise have been resorbed, and thus it accumulates in plasma. An increased [HCO_3^-] without a change in pCO_2(a) increases plasma pH.
            (2) Concurrent hypovolemia and hypochloremia lead to increased renal conservation of HCO_3^-. Hypovolemia stimulates the RAAS to promote HCO_3^- production and H^+ secretion by type A intercalated cells (Figure 9.4). Concurrently, aldosterone activity in principal epithelial cells creates an electrical gradient that also promotes aciduria (Figure 9.2). The aciduria is sometimes called paradoxical aciduria as it occurs with a metabolic alkalosis.
            (3) Cattle in renal failure may develop metabolic alkalosis. The prevailing theory is that the cattle develop abomasal atony that leads to H^+ and Cl^- sequestration, which generates the metabolic alkalosis.

Table 9.9. Disorders and conditions that cause increased serum [HCO_3^-] or [tCO_2] (metabolic alkalosis)

Production of HCO_3^- coupled with loss of H^+ from the body
Gastric production:* vomiting or pyloric obstruction (functional or mechanical)
Renal production
Loop diuretics
Thiazide diuretics
Secondary to respiratory acidosis*
Hypokalemia*
Hyperaldosteronism, primary
Secondary to endurance races or intestinal disorders in horses
Administration of sodium bicarbonate, L-lactate, citrate, magnesium hydroxide, or magnesium oxide in ruminants or horses
Contraction alkalosis:* vomiting, H^+ and Cl^- sequestration, loop diuretics
Metabolic alkalosis perpetuated by hypochloremia
Spurious increase due to increased lactate dehydrogenase activity

* A relatively common disorder, condition, or mechanism

b. Renal production
   (1) Loop diuretics: Furosemide and other loop diuretics (bumetanide and ethacrynic acid) block the action of a Na^+-K^+-$2Cl^-$ transporter; thus, the resorption of Na^+, K^+, and Cl^- by the ascending limb of the LoH is decreased. The resulting impaired H_2O resorption and increased fluid flow in the distal nephron promote H^+ secretion by maintaining a favorable H^+ gradient. Also, hypovolemia and hypochloremia lead to increased secretion of H^+ (see a(2) above) and increased HCO_3^- generation. In addition, increased renal excretion of K^+ may lead to hypokalemia that also promotes H^+ excretion (Figure 9.4).
   (2) Thiazide diuretics: Thiazide diuretics inhibit a Na^+-Cl^- cotransporter in the DCTs by competing for the Cl^--binding site. As noted in the preceding paragraphs, hypovolemia and hypochloremia promote the formation of metabolic alkalosis.
   (3) Secondary to respiratory acidosis: With chronic respiratory acidosis, compensatory renal metabolic alkalosis occurs with increased H^+ secretion by tubular cells (PCTs primarily) and thus increased HCO_3^- generation (Figure 9.8). The compensatory changes in pH and [HCO_3^-] may be apparent within a day of continued respiratory acidosis. The increased H^+ secretion is probably stimulated by the acidemia.
   (4) Hypokalemia: Hypokalemia promotes metabolic alkalosis, alkalemia, or both by multiple methods. When hypokalemia is due to K^+ depletion, it stimulates H^+-K^+-ATPase in the cortical collecting duct (type A intercalated cells), thereby promoting K^+ retention and H^+ secretion (Figure 9.4). Hypokalemia also promotes increased H^+ secretion and HCO_3^- conservation by the PCTs (Figure 9.8 caption).[1] Lastly, hypokalemia alters electrolyte shifts into and out of cells, promoting increases in ICF [H^+] and ECF [HCO_3^-] (Figure 9.5 caption footnote).
   (5) Hyperaldosteronism, primary: Increased aldosterone activity can enhance renal excretion of K^+ to produce hypokalemia (Figure 9.2) that can promote increased production of HCO_3^- (Figure 9.4).

c. Secondary to endurance races or intestinal disorders (e.g., colitis) in horses: Sometimes these alkaloses are interpreted as contraction alkaloses (see Bicarbonate Concentration and Total Carbon Dioxide Concentration, Section III.B.3) or gastric sequestration of H^+ and Cl^-; such mechanisms may be present, but such statements are typically not referenced. Because both conditions produce electrolyte changes that could cause the renal secretion of H^+ and the corresponding generation of HCO_3^-, other mechanisms may be involved.
   (1) Extensive sweating may occur during endurance races. Equine sweat is K^+ rich and Cl^- rich compared to plasma, so sweating results in hypertonic loss of these electrolytes in addition to the loss of body H_2O that may not be replaced by drinking. The resulting hypokalemia promotes renal HCO_3^- generation (see Hypokalemia in the preceding b(4) section).
   (2) Watery diarrhea depletes Cl^- and H_2O. If the horse is not eating, lack of feed intake would reduce tbK^+. As explained in the foregoing Section B.1a(2) and in Figure 9.4, the concurrent states of hypovolemia, hypochloremia, and hypokalemia increase renal excretion of H^+.

2. Administration of sodium bicarbonate, L-lactate, citrate, magnesium hydroxide, or magnesium oxide in ruminants or horses

a. Excess administration of sodium bicarbonate (while treating acidemia) may increase serum [HCO_3^-]. Administration of bicarbonate solutions ("milkshakes") to horses prior to races increases serum [HCO_3^-] and [tCO_2].[142,143]
b. Metabolism of some organic anions (e.g., L-lactate and citrate) by hepatocytes leads to the production of HCO_3^-. Increased [HCO_3^-] is not expected unless large quantities of organic anions are present.
c. Magnesium hydroxide and magnesium oxide administered orally in ruminants cause increased ruminal pH and may cause metabolic alkalosis.[144] In separate studies, administration of magnesium oxide did create a metabolic alkalosis[145] whereas administration of magnesium hydroxide did not.[146] In monogastric animals, there is less of an alkalinizing effect because of the gastric secretion of H^+; in ruminants, the compounds are delivered to the rumen rather than the abomasum.

3. Contraction alkalosis
a. Conceptually, when Cl^--rich but HCO_3^--poor ECF is lost (as may occur with vomiting, H^+ and Cl^- sequestration, and loop diuretics), there is ECF volume contraction without a corresponding drop in HCO_3^- content. Therefore, the [HCO_3^-] in the remaining ECF increases. However, the magnitude of increase from this process is minimal because H^+ is released from other buffers in the body (e.g., PO_4, bone, and Hgb) and combines with the HCO_3^-.
b. Other pathophysiologic processes contribute to a contraction alkalosis.
(1) In response to hypovolemia, aldosterone promotes renal intercalated cell secretion of H^+ and Cl^- accompanied by concurrent production of HCO_3^- (Figure 9.4). Thus, volume contraction promotes alkalosis by increasing renal HCO_3^- production.
(2) The primary processes that result in the loss of Cl^- (vomiting, sequestration, diuretics, and sweating) may also cause the loss of H^+ and coupled production of HCO_3^-, thus contributing to metabolic alkalosis. When there is a loss of Cl^- and accumulation of HCO_3^-, then the Cl^--HCO_3^- exchangers in the gastric parietal epithelial cells (Fig 9.6) or the type A intercalated cells (Figure 9.4) are probably involved in the pathogenesis of the hypochloremic metabolic alkalosis.

4. Metabolic alkalosis perpetuated by hypochloremia: A renal response to alkalemia is to produce H^+ and secrete HCO_3^- via the type B intercalated cells, and this process depends on the presence of Cl^- in the tubular fluid (Figure 9.9). When hypochloremia is present, less Cl^- is available in the tubular fluid and so there is less secretion of HCO_3^- (thus, it accumulates in plasma) and less production of H^+.[1]
5. Spurious increase due to increased serum or plasma LD activity, typically from rhabdomyolysis (see Analytical concepts)

IV. Decreased bicarbonate (HCO_3^-) or total carbon dioxide (tCO_2) concentrations
A. Decreased [HCO_3^-] is a metabolic acidosis, either primary or as compensation for respiratory alkalosis. Metabolic acidoses are typically produced by the generation of excess H^+ or loss of HCO_3^- (Figure 10.6). The loss of HCO_3^- (or other body buffers) reduces the buffering capacity of the body and thus allows H^+ to accumulate and contribute to acidemia. Stated another way, a lower plasma [HCO_3^-] without a corresponding decrease in pCO_2(a) produces a lower plasma pH (as specified by the Henderson–Hasselbalch equation). Additional features of the pathogenesis are explained in Chapter 10.

B. Generally, there are two major types of metabolic acidosis: (i) high-AG metabolic acidosis and (ii) non-AG metabolic acidosis. Calculation of the AG and the diagnostic significance of changes in AG are in a later section of this chapter. More information is presented in Chapter 10 – Mixed acid-base disorders.

C. Disorders and pathogeneses (Table 9.10)

1. Consumption of HCO_3^- after excess generation of H^+: If metabolic pathways generate sufficient H^+ to exceed the buffering capacity of blood, then H^+ accumulates to create acidemia. HCO_3^- is depleted when it is used to buffer the generated H^+. These conditions are called *titrational acidoses* by some authors and may be accompanied by an increased AG.
   a. Lactic acidosis (metabolic acidosis with hyperlactatemia)
      (1) Hyperlactatemia occurs when cellular metabolism relies on glycolysis for generation of ATP or when the degree of glycolysis exceeds the capacity of cells to oxidize the L-lactate that is generated from pyruvate. Acidemia occurs concurrently if aerobic generation of ATP is insufficient to consume the H^+ generated by metabolic processes (see Lactate Concentration in this chapter and section I.F, Acidemia of lactic acidosis, in the Acid-base Abnormalities section of Chapter 10, as well as Figure 10.7).

Table 9.10. Disorders and conditions that cause decreased serum [HCO_3^-] or [tCO_2] (metabolic acidosis)

Consumption of HCO_3^- after excess generation of H^+
Lactic acidosis:[a]* anaerobic cellular respiration, bacterial fermentation of carbohydrates
Ketoacidosis:[a]* excessive lipolysis and fatty acid degradation
Ingestion of certain compounds (Table 9.12)
Consumption of HCO_3^- because of decreased renal/urinary excretion of H^+
Renal failure[a]*
Uroperitoneum or urinary tract obstruction[a]*
Distal renal tubular acidosis (type 1)[b]
Hypoaldosteronism
Consumption of HCO_3^- after excess absorption of H^+
Exogenous PO_4 intoxication from PO_4 enema or urinary acidifier[a]
Increased HCO_3^- loss (commonly with concurrent hyperchloremia)
Alimentary losses:[b]* diarrhea, sequestration, or vomiting of pancreatic secretions
Renal losses:[b] proximal renal tubular acidosis (type 2), inherited or acquired (see text)
Dilutional acidosis (rapid infusion of saline)
Spurious decrease due to aerobic sample handling or underfilling of an evacuated blood collection tube

* A relatively common disorder, condition, or mechanism

[a] Sometimes classified as a titrational acidosis (see Chapter 10, Acid-Base Abnormalities, I.D.1)

[b] Sometimes classified as a secretory (secretional) acidosis (see Chapter 10, Acid-Base Abnormalities, Section I.D.2)

(2) Lactic acidosis can also result from fermentation of carbohydrates by bacteria in ruminants and camelids. The fermentation produces L-lactate, D-lactate, and H^+. Volatile fatty acids may also be produced.
   (a) In adult cattle and in other ruminants, ruminal acidosis (rumen lactic acidosis, grain overload, grain poisoning) occurs when the animals eat excessive amounts of fermentable carbohydrates.
   (b) Similarly, in camelids, excessive ingestion of grains or other fermentable carbohydrates can lead to a forestomach acidosis.
   (c) In neonatal calves with diarrhea, ruminal and enteric fermentation of lactose may be the source of D-lactate and H^+.[147]

b. Ketoacidosis with excessive lipolysis and fatty acid degradation
   (1) The ketosis of ketoacidosis occurs when excessive β-oxidation of fatty acids in hepatocytes causes excessive ketogenesis.
   (2) The acidemia of ketoacidosis occurs from the excess generation of H^+ during lipolysis in adipocytes, and from degradation of liberated fatty acids in hepatocytes (see Figure 10.8 and Acidemia of ketoacidosis in Chapter 10).

c. Ingestion of certain compounds (e.g., ethylene glycol) creates acidemia because the catabolism of the compound generates H^+ (Table 9.12). HCO_3^- is consumed as it buffers H^+.

2. Consumption of HCO_3^- because of decreased renal/urinary excretion of H^+: As with excess generation of H^+, [HCO_3^-] decreases as HCO_3^- (and other buffers) is used to buffer H^+ that accumulates in plasma because it is not being adequately excreted by the urinary system.

a. Renal failure creates acidemia because of the kidneys' inability to excrete the daily acid load produced by metabolic pathways. With progressive renal disease, less NH_4^+ is excreted because there are fewer functional nephrons to form NH_4^+. Abnormalities in HCO_3^- and PO_4 excretion may also play a role in the development of acidemia.

b. Uroperitoneum or urinary tract obstruction
   (1) With uroperitoneum, H^+ is excreted by the kidneys but not from the body, and HCO_3^- is consumed as it buffers the accumulating H^+. Also, urine is typically a HCO_3^--poor fluid, so HCO_3^- shifts down a concentration gradient from plasma to the extravascular space.
   (2) Urinary tract obstructions produce acidemia because of impaired urinary excretion of H^+.

c. Distal renal tubular acidosis (type 1) occurs when there is decreased H^+ secretion by the cells of the collecting ducts. The defect can be specifically in the H^+-ATPase pumps of the collecting ducts. Also, urinary tract obstruction can cause impaired resorption of Na^+ which leads to decreased excretion of H^+ and K^+ in the collecting ducts.[1]

d. Hypoaldosteronism (as seen with hypoadrenocorticism), also called *type 4 renal tubular acidosis*, may promote acidemia through multiple mechanisms:
   (1) Reduced H^+ (and K^+) secretion by principal epithelial cells (Figure 9.2)
   (2) Reduced H^+ secretion by type A intercalated cells (Figure 9.4)
   (3) The resulting hyperkalemia inhibits NH_4^+ excretion in proximal tubules and collecting ducts (Figure 9.7); several mechanisms may be involved.[148] Also, hyperkalemia may cause shifts of H^+ from ICF to ECF (related to concepts in Figure 9.5).

3. Consumption of HCO_3^- after excess absorption of H^+
   a. Some enema solutions contain high concentrations of sodium phosphates (NaH_2PO_4, Na_2HPO_4). After colonic absorption of the sodium phosphates, the phosphates dissociate in ECF with a pH of 7.4 to release Na^+, H^+, $H_2PO_4^-$, and HPO_4^{2-}.[149]
      (1) As HCO_3^- is used to buffer the H^+, [HCO_3^-] decreases.
      (2) Concurrently, hypernatremia, hyperphosphatemia, hypocalcemia, and increased AG are produced.
   b. A metabolic acidosis was found in a cat after it received an excess oral dose of a urinary acidifier containing $NH_4H_2PO_4$, NaH_2PO_4, and $Na_2H_2P_2O_7$.[150] When these compounds dissociate at a pH of 7.4, they release H^+ that is buffered by HCO_3^- to create a metabolic acidosis.
4. Increased HCO_3^- loss (commonly with concurrent hyperchloremia)
   a. Alimentary losses: diarrhea, sequestration, or vomiting of pancreatic secretions
      (1) Intestinal and pancreatic secretions are relatively HCO_3^--rich fluids. Thus, diarrhea, vomiting (if pancreatic secretions are included), and intestinal sequestration can cause HCO_3^- depletion and thus a loss of buffering capacity.
      (2) With time, H^+ produced by metabolic pathways accumulates and promotes acidemia.
   b. Renal losses: proximal renal tubular acidosis (type 2)
      (1) In proximal renal tubular acidosis (type 2), there is a defect in HCO_3^- conservation in the PCTs. The defect may be due to abnormal Na^+ resorption or to the presence of a carbonic anhydrase inhibitor because of treatment or intoxication. The defect may be part of either an inherited or acquired Fanconi syndrome. Besides proximal renal tubular acidosis, other potential findings in Fanconi syndrome include renal glucosuria, aminoaciduria, hypokalemia, and hypophosphatemia.
      (2) Acquired proximal renal tubular acidosis has been seen with multiple myeloma, hypocalcemia, and a variety of drugs in people.
         (a) In dogs, the disorder has been seen with hypocalcemia caused by hypoparathyroidism or hypovitaminosis D, streptozotocin and maleic acid treatments, an overdose of amoxicillin, copper hepatopathy, and in association with ingestion of certain jerky treats.[151–156] Acetazolamide (a diuretic) inhibits carbonic anhydrase activity in the PCT; this can produce a hyperchloremic metabolic acidosis and hypokalemia in dogs.[157,158]
         (b) Acquired proximal renal tubular acidosis of unknown cause has been reported in horses,[159] including two normochloremic quarter horses with mild metabolic acidosis, tubular glucosuria, and aminoaciduria supporting acquired, transient Fanconi syndrome.[160]
5. Dilutional acidosis may occur with rapid saline infusion, which may mildly decrease [HCO_3^-] by diluting ECF HCO_3^-.
6. Spurious decrease due to aerobic sample handling or underfilling evacuated blood collection tubes: in vitro loss of HCO_3^- from the sample (see Bicarbonate Concentration, Section II.B.2)

D. Renal tubular acidoses: Hyperchloremic metabolic acidosis strongly suggests the presence of renal tubular acidosis when there is no evidence of alimentary disease that may cause HCO_3^- loss. In human medicine, there are many disorders or conditions known to create these acidoses and various methods of documenting their presence including quantitative urine chemistry assays and use of urine anion or osmole gaps.[161] (Note: Urine AG is not derived from the same formula as the serum AG.)

1. There is confusion in the literature regarding when it is appropriate to characterize an acidosis as a renal tubular acidosis.
   a. One view is that the classification of renal tubular acidosis should be limited to those disorders in which a renal tubular disorder produces a hyperchloremic metabolic acidosis without an increased AG and there is no evidence of a decreased glomerular filtration rate (i.e., the animal is not azotemic or hyperphosphatemic). Others do not restrict this classification to nonazotemic animals and thus include renal insufficiency or failure patients that might have an increased AG.[162,163]
   b. These differences may be considered relatively unimportant, but the lack of consistency results in confusion and perhaps incorrect case management.
      (1) By the more restrictive definition, the acidosis is created by a tubular dysfunction. That is, either the PCTs or collecting ducts are not adequately secreting H^+ and conserving or producing HCO_3^-. In these animals, plenty of functional glomeruli are providing filtrate to the tubules and there is adequate clearance of urea, creatinine, and phosphates. Diseases that cause loss of functional nephrons should not be considered differentials for such patients.
      (2) In renal failure, the defective renal excretion of H^+ is because of too few functional nephrons. Those nephrons that are still functional can and do secrete H^+. Because the excretion of PO_4 and other anions is also impaired, these animals may have an increased AG.
   c. Some animals may shift from the restrictive group to the nonrestrictive group, i.e., acute proximal tubular disease (e.g., toxicosis) may create proximal renal tubular acidosis, but if the damage is severe enough, the disease may also damage enough nephrons to progress to acute renal failure.
2. For proximal renal tubular acidosis (type 2), proximal tubular disease reduces tubular secretion of H^+ and decreases conservation of HCO_3^-. As shown in Figure 9.8, proximal tubular HCO_3^- conservation normally leads to the movement of HCO_3^- and Na^+ ions from the cells into the interstitial fluid and blood. If HCO_3^- ions are not being formed in cells of the PCTs because of inhibition of carbonic anhydrase, the Na^+-K^+-ATPase pump creates an electrical gradient that enhances Cl^- resorption. The net result in plasma is decreased [HCO_3^-], increased [Cl^-], and therefore hyperchloremic metabolic acidosis.
3. For distal renal tubular acidosis (type 1), collecting duct disease impairs the secretion of H^+ and thus decreases the generation of HCO_3^- by the carbonic anhydrase reaction. As shown in Figure 9.4, decreased generation of HCO_3^- by the type A intercalated epithelial cells decreases the renal secretion of Cl^-. The net result in plasma is decreased [HCO_3^-], increased [Cl^-], and therefore hyperchloremic metabolic acidosis. Anticonvulsants with carbonic anhydrase inhibitory properties (e.g., zonisamide, topiramate) have been associated with distal or mixed renal tubular acidosis in dogs, cats, and people.[164]
4. Type 4 renal tubular acidosis is the result of selective aldosterone deficiency or resistance. The major actions of aldosterone are to promote Na^+ resorption and K^+ and H^+ secretion; thus, diminished activity leads to hyperkalemia and mild acidemia. The hyperkalemia contributes to the acidemic state by impairing NH_4^+ production and secretion in the nephrons.[148] (Note: Selective aldosterone deficiency is different from hypoadrenocorticism in which there is impaired production of cortisol and aldosterone.)
5. Type 3 renal tubular acidosis is rarely reported in people; it is considered a variant of type 1 or, occasionally, a combination of type 1 and type 2 renal tubular acidosis.

ANION GAP

I. Definitions
   A. *Cation*: an atom or molecule with a positive charge; monovalent cations have one positive charge and divalent cations have two
   B. *Measured cation charge* (mC^+): the charge contributed by the major monovalent cations whose serum or plasma activities or concentrations are directly measured (Na^+ and K^+); because these ions are monovalent and measured as free ions, their ion concentrations are equivalent to their charge concentrations
   C. *Unmeasured cation charge* (uC^+): the charge contributed by all other cations in blood, including fCa^{2+}, fMg^{2+}, and cationic globulins, for which concentrations are not measured for the AG calculation (but may be measured); charge concentrations for cations that are not monovalent are greater than their ion concentrations
   D. *Total cation charge* (tC^+): the total charge of all cations in the blood ($tC^+ = mC^+ + uC^+$)
   E. *Anion*: an atom or molecule with a negative charge; monovalent anions have one negative charge, divalent anions have two, and trivalent anions (e.g., PO_4^{3-}) have three
   F. *Measured anion charge* (mA^-): the charge contributed by the major monovalent anions whose serum or plasma activities or concentrations are measured (Cl^- and HCO_3^-); because these ions are monovalent and measured as free ions, their ion concentrations are equivalent to their charge concentrations (Note: $[HCO_3^-]$ may be estimated from $[tCO_2]$.)
   G. *Unmeasured anion charge* (uA^-): the charge contributed by all other anions in serum, including PO_4, albumin, anions of organic acids, and SO_4, for which concentrations are not measured for the AG calculation (though some, such as PO_4 and L-lactate may be measured); charge concentrations for anions that are not monovalent are greater than their ion concentrations
   H. *Total anion charge* (tA^-): the total charge of all anions in the blood ($tA^- = mA^- + uA^-$)
   I. *mmol/L of ion charge*: the positive or negative electrical charge of an electrolyte per liter, equal to milliequivalent per liter (mEq/L)
   J. *mEq/L*: An equivalent is the number of moles of an ion in solution multiplied by the valence of that ion; a milliequivalent per liter is one-thousandth of the number of moles of ion charge per liter (i.e., mmol/L of ion charge). (Further definition: An *equivalent* is the amount of a substance that reacts with – is equivalent to – a known amount of another substance in a chemical reaction, i.e., the amount supplying 1 mole of either H^+ in an acid-base reaction or electrons in a redox reaction.)
   K. *Anion gap* (AG): AG is the difference (gap) in the charge concentrations (not ion concentrations) between uA^- and uC^+ ($AG = [uA^-] - [uC^+]$). The AG can be calculated because this is also equal to the difference between $[mC^+]$ and $[mA^-]$ (Eq. 9.6a.). This difference (the AG) is positive in health, reflecting more uA^- than uC^+. Contributors to $[mC^+]$ are $[Na^+]$ and $[K^+]$, and contributors to $[mA^-]$ are $[Cl^-]$ and $[HCO_3^-]$; these are used to calculate the AG (Eq. 9.6b). Because $[K^+]$ rarely varies more than 2 mmol/L from RI, some people prefer a formula without $[K^+]$ (Eq. 9.6c). (Note: RIs for the two calculated AGs differ.). The AG equations are often written and discussed in terms of "measured anions" and "measured cations" rather than in terms of the charges of unmeasured anions and cations. Such an approach works when considering only the monovalent electrolytes whose charge and mass concentrations are equivalent, but the role of charge is important to consider in understanding the effects of polyvalent cations and anions (e.g., Ca^{2+}, PO_4, albumin).

Perhaps the calculated value should be called the "anionic-charge gap" or, because the gap is in units of concentration, the "anion-charge gap concentration."

$[mC^+] + [uC^+] = [mA^-] + [uA^-]$ because serum is electrically neutral ($[tC^+] = [tA^-]$).
Rearranging: The gap in charge concentrations = $[uA^-] - [uC^+] = [mC^+] - [mA^-]$
Thus, $AG = [mC^+] - [mA^-]$ (**9.6a**)

Because $[mC^+] = [Na^+] + [K^+]$ and $[mA^-] = [Cl^-] + [HCO_3^-]$,

$$AG = ([Na^+] + [K^+]) - ([Cl^-] + [HCO_3^-]) \quad (\textbf{9.6b})$$

$$\text{Variant equation: } AG = [Na^+] - ([Cl^-] + [HCO_3^-]) \quad (\textbf{9.6c})$$

II. Physiologic processes
   A. Plasma is always electrically neutral, i.e., total positive charges equal total negative charges. The major contributors to the electroneutrality and their relative contributions are listed in Table 9.11. In the example, 150 of the 157 mmol/L of cation charge is from Na^+ and K^+ (the mC^+), and 134 of the 157 mmol/L of anion charge is from Cl^- and HCO_3^- (mA^-).
   B. The major purpose of calculating AGs is to identify increased $[uA^-]$ and therefore an increase in circulating anionic molecules (e.g., L-lactate and ketone bodies) in states of metabolic acidosis (Figure 9.10).

Table 9.11. Cation and anion charge concentrations of plasma or serum in health (approximate measured concentrations provided to simplify concept)[a]

Cations	mmol/L ion charge[b]	Anions	mmol/L ion charge[b]
Na^+	146	Cl^-	110
K^+	4.0	HCO_3^-	24
fCa^{2+}	5.0	Proteins	16[c]
fMg^{2+}	2.0	Organic anions	3.5[d]
H^+	0.00004	PO_4	2.5
		SO_4	1.0
Total	157	Total	157

[a] The provided ionic charge values are the clinically measured concentrations and not the true electrical charge contributions of the analytes because a small portion of the electrolytes exists as molecules, e.g., NaCl, $NaHCO_3$. Also, about half of the PO_4 anions are bound to plasma cations and thus are not contributing to ionic charge.

[b] mmol/L ion charge is another way of expressing mEq/L

[c] Molar concentrations of proteins are not measured because of variations in molecular weights of proteins. (Note: Proteins are the major component of the AG in healthy animals.)

[d] In health, organic anions (e.g., L-lactate, AcAc, BHB, and citrate) contribute very little to tA^- because their molar concentrations are relatively small compared to those of the other anions. However, they are frequently the reason for an increased AG when they accumulate in plasma, i.e., when there is hyperlactatemia or hyperketonemia.

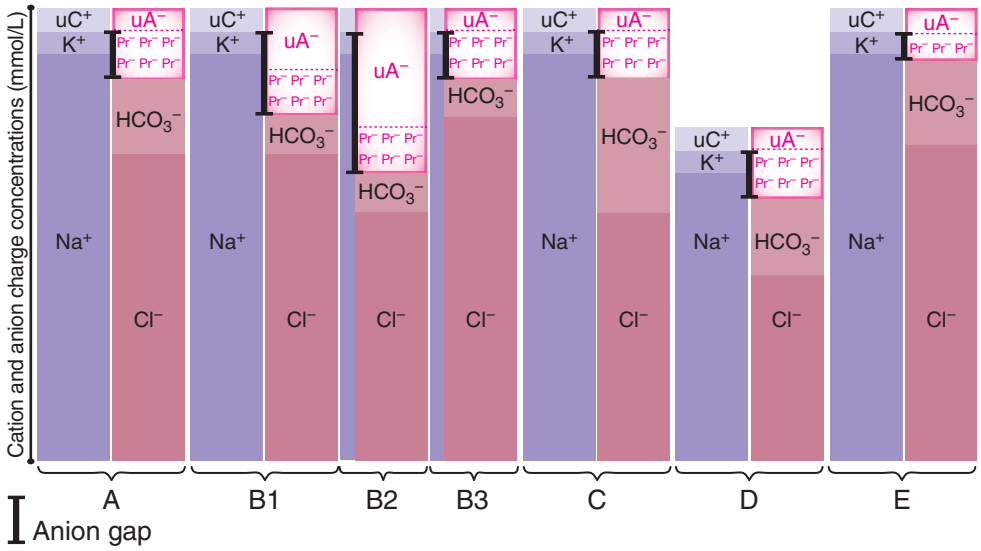


Figure 9.10. Bar diagrams of anion gap concepts.
In all cases, total cation charge concentration equals total anion charge concentration. The uC^+ is due mostly to charges from fCa^{2+}, fMg^{2+}, and some globulins, whereas the uA^- is due mostly to charges from PO_4, SO_4, anions of organic acids (e.g., lactate, AcAc), and proteins (Pr^-), particularly albumin. Proteins are separated in the diagrams to illustrate their relative contributions to tA^-. In these examples, the uC^+ concentrations remain constant, but they may be altered in pathologic states.

A. Healthy animals: The AG is almost equivalent to the combined physiologic charge concentrations of anionic proteins.

B. Metabolic acidoses (partial bars of cations shown in B2 and B3):
B1. Normochloremic metabolic acidosis (low $[HCO_3^-]$): The increased AG represents increased concentrations of uA^- from organic (e.g., lactate) or inorganic (e.g., PO_4, SO_4) anions associated with organic (e.g., lactic acidosis) or inorganic (e.g., renal failure) acidoses.
B2. Hypochloremic metabolic acidosis (low $[HCO_3^-]$ and low $[Cl^-]$): The increased AG represents increased concentrations of uA^- from organic acid anions, PO_4, SO_4, or other anions besides Cl^- or HCO_3^-. These acidoses may be organic (e.g., lactic acidosis) or inorganic (e.g., renal failure).
B3. Hyperchloremic metabolic acidosis (low $[HCO_3^-]$ and high $[Cl^-]$): The AG typically is not increased. These acidoses are due to renal tubular acidoses (proximal or distal) and diarrheas.

C. Hypochloremic metabolic alkalosis (high $[HCO_3^-]$): The AG is not increased because the sum of $[Cl^-]$ and $[HCO_3^-]$ and the sum of $[Na^+]$ and $[K^+]$ have not changed. These alkaloses may occur with vomiting or gastrointestinal sequestration of H^+ and Cl^-.

D. Concurrent hyponatremia and hypochloremia: The AG has not changed because the $[Na^+]$ and $[Cl^-]$ decreased proportionately and other concentrations did not change. These findings are more common when there is concurrent loss of Na^+ and Cl^-, e.g., with intestinal loss via diarrhea or renal loss in hypoadrenocorticism.

E. Hypoproteinemia (particularly hypoalbuminemia): The AG is decreased because there is a lower concentration of proteins, an increased sum of $[Cl^-] + [HCO_3^-]$, and no change in the sum of $[Na^+] + [K^+]$. These findings occur in hypoproteinemic disorders when there are not concurrent acid-base disorders (e.g., hepatic failure). Increased $[HCO_3^-]$ with hypoproteinemia indicates that there is a metabolic alkalosis.

1. As shown in Table 9.11, $uA^- > uC^+$ in health, i.e., the sum of charges due to proteins, organic ions, PO_4, and SO_4 is greater than the sum of charges due to fCa^{2+}, fMg^{2+}, and H^+ (~23 mmol/L vs 7 mmol/L). Also, the "unmeasured" anion concentrations can change more markedly than can the "unmeasured" cation concentrations.

2. Clinically significant changes in the AG are usually the result of changes in uA^-. Major changes in $[fCa^{2+}]$ and $[fMg^{2+}]$ are life threatening. Only minor changes in $[fCa^{2+}]$ and $[fMg^{2+}]$ are seen clinically, and these cause only minor changes in AG.
3. Most changes in AG are due to increased concentrations of the anions of organic acids. These anions may be endogenous substances (e.g., L-lactate, AcAc, and BHB), or they may be generated from exogenous substances (e.g., metabolites of ethylene glycol).
4. Increases and decreases in protein concentrations, especially albumin, cause increases and decreases, respectively, in the AG. Most plasma proteins are in the uA^- group. Hyperglobulinemia caused by paraproteinemia may increase the AG when the paraprotein is negatively charged and decrease the AG when it is positively charged.[165] Marked hyperglobulinemia (or lipemia) causing pseudohyponatremia and pseudohypochloremia would yield inaccurate AG values (always falsely decreased) but only mildly inaccurate because of proportional effects on $[Na^+]$ and $[Cl^-]$.
5. With hyperphosphatemia, each 1 mg/dL increase in [Pi] is equal to 0.3229 mmol/L Pi, which is equal to 0.3229 mmol/L PO_4. In serum or plasma, PO_4 has an average negative valence near 1.8 (4 parts $H_2PO_4^{2-}$ to 1 part $H_3PO_4^-$). Thus, a 1 mg/dL increase in [Pi] potentially creates an anionic charge of about 0.6 mmol/L ($0.3229 \times -1.8 \cong -0.6$) or −0.6 mEq/L. However, only about half of the PO_4 exists as free anions (0.3 mmol/L) in the healthy animal, and PO_4 bound to protein and nonprotein cations does not contribute to anionic charge. So, every 5 mg/dL increase in [Pi] can explain about a 1.5 mmol/L (5×0.3) increase in AG. With marked hyperphosphatemia, a greater portion of total PO_4 may exist as free anions.

III. Analytical concepts
   A. Units: mmol/L of ion charge, the same as mmol/L of ion or mEq/L of ion for monovalent ions, but not equivalent to ion concentration for multivalent ions like PO_4, fCa^{2+}, and albumin (mmol/L of ion charge is equal to mEq/L; "mmol/L of ion charge" is used in this chapter because the SI unit for electrolytes is mmol/L)
   B. As with any calculated value, the AG is only as accurate as the measured values. If needed, refer to prior sections on the analytical aspects of individual electrolytes.
   C. The $[HCO_3^-]$ used in the formula may be a $[tCO_2]$, a calculated $[HCO_3^-]$, or a measured $[HCO_3^-]$. Even with marked elevations in pCO_2(a) values, the tCO_2 and HCO_3^- values do not differ by more than 2 mmol/L (2 mEq/L) if both values are accurate. An AG should be interpreted relative to a RI generated with the same instruments, tests, and formula.

IV. Increased AG
   A. This typically is seen with certain metabolic acidoses (especially normochloremic and hypochloremic), but an increased AG is not specific for a particular cause. Hyperchloremic metabolic acidoses typically do not have increased AG concentrations. Minor increases in the AG concentration can be seen in nonacidotic disorders that cause hyperproteinemia or reduce the concentration of fCa^{2+} and fMg^{2+}.
   B. Because the AG is a calculated value, the value may be erroneous if any of the values used to calculate it are erroneous.
      1. Pseudohyponatremia and pseudohypochloremia would lead to mildly low AG values. Depending on the cause of the H_2O displacement, $[K^+]$ and $[HCO_3^-]$ may also be erroneous.
      2. The AG will be falsely decreased if the $[Cl^-]$ is falsely increased (e.g., with bromide or excess heparin in the sample).

3. The AG will be falsely increased when [HCO_3^-] is falsely decreased because of escape of CO_2 from serum or plasma samples excessively exposed to air or because evacuated tubes were incompletely filled.
4. A marked increase in apparent [K^+] due to a pseudohyperkalemia will also increase the AG. A true hyperkalemia does not cause an increased AG because electroneutrality requires corresponding changes in Na^+, Cl^-, or HCO_3^- concentrations.

C. When "What causes the increased AG?" is asked, the question can be answered two basic ways.
1. Mathematically: Because serum or plasma must be electrically neutral (i.e., total cation charge = total anion charge), a decreased sum of [Cl^-] + [HCO_3^-] without a change in [tC^+] requires an increased [uA^-]. Thus, from a mathematic perspective and for preservation of electroneutrality, a decreased sum of [Cl^-] + [HCO_3^-] "causes" the increased [uA^-] and AG. However, physiologic processes do not directly trade uA^- for either Cl^- or HCO_3^-.
2. Pathophysiologically: Except for the minor changes associated with decreased [fCa^{2+}] or [fMg^{2+}], increased AG indicates increased [uA^-], and therefore increased concentration of anions other that Cl^- and HCO_3^-. The pathogenesis of the increased AG therefore is the pathogenesis of the increased [uA^-], and the changes in [Cl^-] + [HCO_3^-] are just secondary reflections of the disorder that must occur to maintain electroneutrality. The increased [uA^-] can be due to increased concentrations of one or more types of anions.

D. Disorders and conditions that increase the AG are listed in Table 9.12.

E. Pathogeneses of increased AGs (assuming measured concentrations are valid)

Table 9.12. Disorders and conditions that cause an increased AG

Metabolic acidoses
Lactic acidosis:* increased [lactate] (either L-lactate or D-lactate)
Ketoacidosis:* increased ketone bodies ([BHB] or [AcAc], not acetone)
Renal failure:* increased [PO_4], [SO_4], [citrate], and other organic anions
Exogenous PO_4 intoxication from PO_4 enema or urinary acidifier
Cobalamin deficiency causing methylmalonic aciduria in dogs
Massive rhabdomyolysis: probably increased [L-lactate] and [PO_4]
Ingestion of certain compounds
Ethylene glycol (antifreeze):* increased [glycolate] or [oxalate]
Methanol poisoning (antifreeze): increased [formate]
Paraldehyde (sedative or anesthetic): increased [acetate] and [chloroacetate]
Metaldehyde poisoning (snail bait)
Hyperalbuminemia and possibly inflammatory hyperglobulinemia (minor changes)
Increased concentrations of certain anionic paraproteins
Spurious increases cause by falsely decreased [HCO_3^-], pseudohypochloremia, or pseudohyperkalemia

* A relatively common disorder, condition, or mechanism

Note: Concurrent hypoalbuminemia and potentially other causes of decreased AG (Table 9.13) can reduce or mask increases in AG caused by these disorders and conditions.

1. Metabolic acidoses
   a. Lactic acidosis and ketoacidosis: In lactic acidosis or ketoacidosis (each with or without hypochloremia), the pathogenesis of the increased AG (i.e., increased [uA^-]) is the pathogenesis of the hyperlactatemia (see later section) or hyperketonemia (see later section), respectively. The pathogenesis of the decreased [HCO_3^-] is consumption as it buffers the excessive production of H^+ (see lactic acidosis and ketoacidosis in Chapter 10). If present, multiple factors could contribute to a hypochloremia; a common factor is increased renal excretion of Cl^- in response to acidemia. With lactic acidosis, the pathogeneses of the hyperlactatemia, decreased [HCO_3^-], and hypochloremia have common initiating events, but the hyperlactatemia does not cause the decreased [HCO_3^-] or hypochloremia (or vice versa). With ketoacidosis, the pathogeneses of the hyperketonemia, decreased [HCO_3^-], and hypochloremia have common initiating events, but the hyperketonemia does not cause the decreased [HCO_3^-] or hypochloremia (or vice versa).
   b. In the metabolic acidosis of renal failure, the pathogenesis of the increased AG (i.e., increased [uA^-]) is the pathogenesis of the increased concentrations of PO_4, SO_4, citrates, and other organic anions, i.e., decreased renal excretion of these anions because of decreased glomerular filtration rate. The [HCO_3^-] is decreased because it is consumed as it buffers the H^+ that is not excreted adequately by renal tubules.
   c. Exogenous PO_4 intoxication causing marked hyperphosphatemia
      (1) Marked hyperphosphatemia and metabolic acidosis may occur after administration of PO_4 enemas in cats and dogs[20,21] or ingestion of a PO_4 urinary acidifier.[150]
      (2) To determine if hyperphosphatemia is the major contributor to an increased AG, the [PO_4] need to be expressed in mEq/L or in mmol ionic charge/L (see Physiologic processes II.B.5). In addition to increased PO_4 in enema cases, increased [albumin] (from H_2O shifting to colon) and decreased [fCa^{2+}] (from formation of Ca^{2+}-PO_4 compounds) may contribute to an increased AG.
   d. When there is a marked cobalamin deficiency that causes methylmalonic aciduria in dogs with intestinal disease, a small percentage of the affected dogs can have increased serum concentrations of anions from methylmalonic acid, and those anions may cause an increased AG.[166,167] Increased serum [methylmalonic acid] also occurs in dogs with hypocobalaminemia from cobalamin receptor defects.[168]
   e. Massive rhabdomyolysis: An increased AG may be found in horses with rhabdomyolysis, probably due to increased [L-lactate] (from anaerobic respiration) and [PO_4] (from myocytes).
   f. Ingestion of certain compounds: As listed in Table 9.12, metabolism of certain compounds results in production of anions other than Cl^- or HCO_3^-, and these anions can cause an increased AG.
2. Hyperalbuminemia and possibly inflammatory hyperglobulinemia (anionic globulins): These proteins contribute to the AG seen in healthy animals. When their concentrations increase, then the AG increases.
3. Increased concentrations of certain anionic paraproteins: In rare paraproteinemias caused by lymphoid neoplasms, the abnormal protein may carry enough negative charge to increase the AG.
4. Spurious increases caused by falsely decreased [HCO_3^-], pseudohypochloremia due to decreased [HCO_3^-], or pseudohyperkalemia

a. Falsely low [HCO_3^-] due to sample exposure to air is a common reason for a pseudo–metabolic acidosis with increased AG.
b. Hypochloremia due to decreased [HCO_3^-] would also cause an increased AG (see Cl^- analytical section) (Note: Pseudohypochloremia due to lipemia or marked hyperproteinemia would actually lead to a mildly lower AG because the error in the concurrent pseudohyponatremia is greater than the pseudohypochloremia error).
c. Pseudohyperkalemia cause by hemolysis or thrombocytosis would slightly increase the AG.

V. Decreased AG
A. A decreased AG, which signals the presence of either decreased "unmeasured" anions or increased "unmeasured" cations, is of little direct diagnostic value because such changes are typically identified by other means.
1. Hypoalbuminemia (decreased uA^-) is a common cause of decreased AG, but hypoalbuminemia is evident directly from the [albumin]. Decreases in the concentrations of other "unmeasured" anion charges can only mildly decrease the AG because their concentrations are too low in health to decrease substantially.
2. Increases in uC^+ (e.g., fCa^{2+}, fMg^{2+}, and certain paraproteins) are recognized directly by measurement of fCa^{2+}, fMg^{2+}, and protein concentrations, so a decrease in AG is not needed as a clue to their presence.
B. Recognizing common factors like hypoalbuminemia that lower the AG aids in the interpretation of AG values that are WRI or increased; the AG value would have been greater if the animal had not had hypoalbuminemia.
1. Studies in people suggest that each 1 g/dL decrease in [albumin] equates to about a 2.3–2.5 mmol/L decrease in AG.[169]
2. In contrast, an estimate for the negative charge contribution of albumin in dogs was 4.2 mmol/L for every 1 g/dL albumin.[170] Theoretically then, if an assay's RI is 3.0–3.8 g/dL and a dog's [albumin] is 1.4 g/dL (2 g/dL below the midpoint of the RI), hypoalbuminemia would lower the AG by about 8 mmol/L (4.2 mmol/L × 2).
3. Because there is not consistent agreement in albumin assays and there are species differences in the charge of albumin molecules, any relationship between [albumin] and AG is assay dependent and likely species specific. Estimates of expected effects of albumin concentrations on the AG should be based on RIs generated by the same assay used for a patient. It should also be noted that the relationship does not include the contributions of changes in [globulins] to the AG.
C. AG calculations may yield negative numbers, usually because of errors in measurement (e.g., falsely increased [Cl^-] because of bromide in the sample or falsely increased [tCO_2] or [HCO_3^-] because of LD interference). Rarely, paraproteinemia has been associated with a negative AG;[171] concurrent hypoalbuminemia would contribute. Other potential causes of a negative AG in people have been reported.[172]
D. Disorders and conditions that decrease the AG are presented in Table 9.13.

VI. Strong-ion difference (SID)
A. Evaluation of SID assesses acid-base abnormalities in the context of abnormal concentrations of ions (strong cations and strong anions). SID and strong-ion gap concepts are similar to AG concepts but also include consideration of other factors.
B. Major aspects of SID are presented in Chapter 10.

Table 9.13. Disorders and conditions that cause a decreased AG
Decreases in uA^-
Hypoalbuminemia*
Increases in uC^+
Hypercalcemia (minor changes)
Hypermagnesemia (minor changes)
Multiple myeloma that is producing cationic proteins
Spurious decrease caused by pseudohyperchloremia (e.g., bromide interference) or falsely increased [tCO_2] or [HCO_3^-] (e.g., LD interference)[a]

* A relatively common disorder, condition, or mechanism

[a] Marked hyperlipidemia may also falsely lower the AG by causing proportionately greater pseudohyponatremia than pseudohypochloremia, but the effect is mild and unlikely to cause a decreased AG alone.

LACTATE CONCENTRATION (L-LACTATE AND D-LACTATE)

I. Physiologic processes

A. The glycolytic pathway anaerobically converts glucose into energy (ATP) and generates pyruvate in aerobic and anaerobic states. Traditional thought is that the pyruvate formed in hypoxic cells is converted to L-lactate, whereas pyruvate formed without hypoxia enters the Krebs cycle in mitochondria to generate energy via oxidative phosphorylation. However, there is a body of evidence suggesting that most of the pyruvate generated by glycolysis is converted to L-lactate whether conditions are aerobic or anaerobic, and, depending on the state of the cell, the generated L-lactate is then transported either (i) into mitochondria where LD converts it to pyruvate for subsequent oxidative energy generation or (ii) out of the cell and through interstitial fluid or blood for entry into cells that are nearby (e.g., from myocyte to myocyte, from astrocyte to neuron) or more distant (e.g., from skeletal muscle cells to cells in the brain or heart), thus providing a source of energy and cell signals elsewhere.[173] Whenever glycolysis increases (e.g., with muscle activity or epinephrine), L-lactate generation increases (Figure 9.11). However, plasma [L-lactate] depends not only on L-lactate production but also on the degree of L-lactate consumption and its net flux into and out of cells.

B. The major tissue source of L-lactate in health is skeletal muscle, but it is also produced by blood cells and cells in many other tissues, including heart, brain, liver, kidney, intestine, adipose tissue, and lung.[174] L-lactate is used for gluconeogenesis in the liver and kidneys or for ATP production via the Krebs cycle in these organs and many others. Hepatocytes take up most plasma L-lactate; excess is excreted in urine (Figure 9.12).

C. Because mammalian RBCs lack mitochondria, L-lactate produced by glycolysis in RBCs must move to plasma. RBCs also act as a sink for blood L-lactate, taking up L-lactate from muscle or other cells. L-lactate may enter or exit RBCs in three ways: (i) facilitated diffusion of L-lactate via bidirectional H^+-linked monocarboxylate transporters, (ii) simple diffusion of lactic acid across cell membranes, and (iii) L-lactate exchange with Cl^- or HCO_3^- via the inorganic anion-exchange transporter (band-3 protein).[175,176] The relative contributions of these three methods varies among and within species. After exercise, as much as ~50 % of blood L-lactate can be within equine RBCs, but, even then, the [L-lactate] within RBCs is similar to or lower than plasma [L-lactate], so hemolysis is not expected to cause clinically significant false increases of plasma [L-lactate].

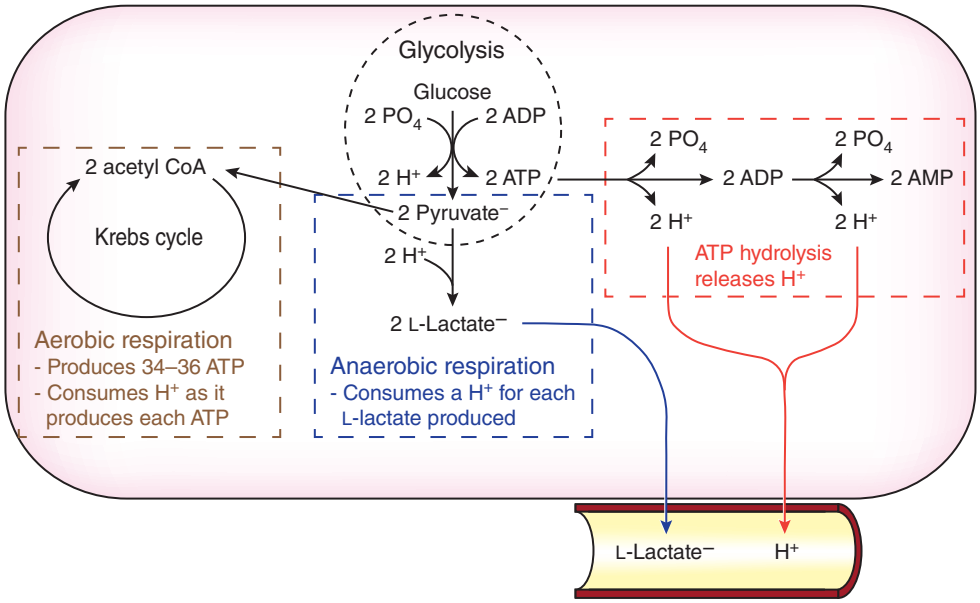


Figure 9.11. Lactate-associated acidosis (lactic acidosis).
During states of impaired aerobic respiration (hypoxia) or accelerated glycolysis, when cells produce increased amounts of L-lactate, more H^+ may be released during ATP use (hydrolysis) than is consumed by aerobic ATP generation through the Krebs (citric acid, tricarboxylic acid) cycle and oxidative phosphorylation. The generated L-lactate and H^+ enter blood and promote hyperlactatemia and acidemia, respectively.

- Glycolysis: Cellular catabolism of a glucose molecule produces two pyruvate molecules plus a net yield of two H^+ ions and two ATP molecules. Therefore, glycolysis yields relatively little ATP from each glucose molecule. With hypoxia, pyruvate is reduced to L-lactate; this may also occur under aerobic conditions. Reduction of pyruvate is catalyzed by LD in a reaction that consumes H^+, so there is no net generation of H^+ in the formation of L-lactate through glycolysis.
- Aerobic respiration: Either pyruvate or L-lactate enters mitochondria for aerobic respiration, with L-lactate requiring mitochondrial conversion back to pyruvate for further metabolism. Pyruvate dehydrogenase, a thiamine-dependent enzyme, converts pyruvate to acetyl CoA which enters the Krebs cycle to support ATP generation through oxidative phosphorylation. With adequate oxidative capacity and cell oxygenation, each pyruvate molecule generates 17–18 molecules of ATP (34–36 per glucose molecule). In the process, the H^+ produced by ATP hydrolysis for energy-requiring cellular functions is consumed.
- Insufficient aerobic respiration: When cellular hypoxia is present or if aerobic respiration cannot keep up with an increased degree of glycolysis, L-lactate is released to plasma (Fig 9.12). The pK_a of lactic acid is 3.86, so nearly all of the L-lactate produced remains as such; very little buffers H^+ to form lactic acid at physiologic pH values.
- ATP hydrolysis: As explained in Chapter 10 (Lactic acidosis), the acidemia of lactic acidosis is due to H^+ release during hydrolysis of ATP for energy, not release from glycolysis, and it builds up when H^+ is not consumed by aerobic respiration.

ADP, adenosine diphosphate; AMP, adenosine monophosphate; CoA, coenzyme A

D. Bacteria produce D-lactate, and some produce L-lactate. At least some protozoa generate and release lactate; *Plasmodium* produces mostly L-lactate but also some D-lactate. The mirror-image stereoisomer of L-lactate, D-lactate, can also be produced in mammalian cells. This can occur via the *glyoxalase pathway*, which metabolizes methylglyoxal produced during glycolysis or from the metabolism of acetone.[177] Some mammals can generate D-lactate from D isomers

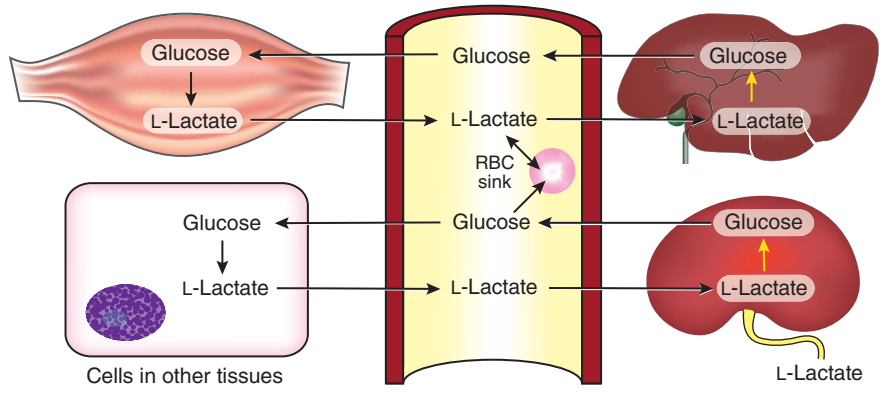
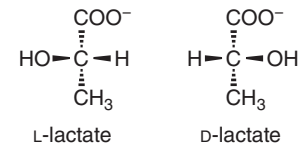


Figure 9.12. L-Lactate metabolism.
The traditional view is that pyruvate is the end product of glycolysis, and it is shuttled to mitochondria for oxidative metabolism, whereas L-lactate is a glycolytic waste product formed mostly through anaerobic metabolism. However, evidence suggests that L-lactate is the main product of glycolysis under aerobic and anaerobic conditions, and the main metabolic intermediate that is shuttled into mitochondria for ultimate oxidative generation of ATP. In either case, with increased glycolysis in health or disease, L-lactate not used to generate energy in a cell can be transported to blood, and from there it can enter other cells (e.g., in the heart and brain) for oxidative metabolism. In addition, L-lactate can be used for gluconeogenesis by hepatic and renal cells, and the generated glucose can enter blood for use by cells in other tissues.

- In the Cori cycle, when L-lactate begins to accumulate in an active myocyte because its generation exceeds the cell's capacity for oxidative metabolism, L-lactate is shuttled through membrane transporters down a concentration gradient to plasma, and then taken up by hepatocytes. In hepatocytes, L-lactate is converted to glucose (gluconeogenesis), which then enters blood and cycles back to muscle (or is used in other cells). Similarly, substantial gluconeogenesis from L-lactate occurs in epithelial cells of renal PCTs, and the generated glucose can enter blood to be used by myocytes or cells in tissues throughout the body. This can also be considered part of the Cori cycle. In this way, gluconeogenesis supports glucose needs of other cells, such as active myocytes that cannot generate enough glucose from their own pathways of glycogenolysis and glycolysis.
- Although many cells can produce and metabolize L-lactate for energy, gluconeogenesis is limited to the liver and kidneys.
- Besides producing L-lactate, RBCs take up L-lactate from plasma, thereby serving as a "lactate sink" that lowers plasma [L-lactate], thus facilitating diffusion of L-lactate down a concentration gradient from cells to plasma. Mammalian RBCs cannot use L-lactate for energy; they are limited to glycolysis, and in most mammals (pigs are an exception), glucose is the sole fuel for RBCs.
- When L-lactate formation and transport into plasma exceed its consumption and uptake by cells, it is excreted in urine.

of exogenous propylene glycol via alcohol and aldehyde dehydrogenases rather than the glyoxylase pathway. In health, plasma concentrations of D-lactate are very small compared to those of L-lactate.



lactate isomers (mirror images)

II. Analytical concepts
A. L-Lactate

1. Common clinical assays for lactate measure the L-lactate – not D-lactate – produced by animal cells. L-Lactate assays use either LD (spectrophotometric methods) or L-lactate oxidase (e.g., i-STAT and NOVA). The analytical accuracy and precision of point-of-care instruments vary.[178–181]
2. L-Lactate concentrations are measured by many blood gas analyzers. Depending on the analytical method, either whole blood or plasma is the preferred sample. In the NOVA instruments, plasma [L-lactate] is measured in a whole blood sample by allowing only plasma to enter the reaction chamber.

B. D-Lactate concentrations can be measured by high-performance liquid chromatography or by D-lactate dehydrogenase enzymatic methods. These assays are not commonly available in clinical laboratories, but automated assays have been developed. Assay validation should include data demonstrating that increased [L-lactate] and LD activity do not interfere.

C. Unit conversion: mg/dL × 0.112 = mmol/L (SI unit); the conversion factor for lactic acid is 0.111, but the pK_a of lactic acid is 3.86 and thus the ratio of L-lactate to L-lactic acid is > 1000:1 at a pH of 7.4

D. Sample for [L-lactate]
1. Blood samples should be processed or analyzed quickly so that L-lactate produced by RBCs does not increase the plasma [L-lactate]. If the sample cannot be processed immediately, L-lactate production can be reduced by collecting blood into a tube containing sodium fluoride and chilling the blood until the plasma is separated from the RBCs; fluoride inhibits phosphopyruvate hydratase (synonym: enolase) of the glycolytic pathway, thus blocking pyruvate and L-lactate formation. The low pH of certain sodium fluoride tubes containing citrate and EDTA additionally inhibits early glycolytic enzymes. If EDTA- or heparin-anticoagulated whole blood sits at room temperature for an hour, artifactual increases in [L-lactate] may be close to 1 mmol/L.
2. Ideally, free-flowing blood should be collected (minimal use of a tourniquet) so that blood stagnation does not result in increased L-lactate production.
3. L-Lactate concentrations are also measured in body cavity effusions as evidence of bacterial infection (see Chapter 19).
4. Special collection recommendations for D-lactate samples were not found, but plasma deproteinization may be required prior to analysis.

E. Substances that interfere with the measurement of [L-lactate] vary with the method used.
1. Bromide falsely decreases the [L-lactate] determined by the i-STAT L-lactate oxidase method, but no clinically significant interference was reported for 37.5 mmol/L bromide with the NOVA L-lactate oxidase method (user manual).
2. Free hemoglobin and hemoglobin-based oxygen carriers (e.g., hemoglobin glutamer or hemoglobin raffimer) in the sample falsely lower [L-lactate] if measured by the L-lactate oxidase assay.[182,183] In the presence of hemoglobin glutamer, the measured concentrations were decreased 3–13 % using various instruments; hemoglobin raffimer had less effect.[183] The falsely low concentrations are probably due to the peroxidase activity of heme, which removes the H_2O_2 produced by the L-lactate oxidase reaction.

III. Increased plasma L-lactate concentrations (*hyperlactatemia*)

A. L-Lactate accumulates in plasma when its formation by tissue cells or organisms in the alimentary tract exceeds the removal by tissues (primarily by hepatocytic uptake and passage into the renal filtrate).[174] Hyperlactatemia is commonly associated with metabolic acidosis and increases in the AG. However, not all causes of hyperlactatemia generate excess H^+ leading to decreased [HCO_3^-] (i.e., metabolic acidosis).

B. [L-Lactate] is greater in healthy neonatal foals (1–3 d) than in adult horses.[184] Reported mean concentrations are about 4 mmol/L but were as high as 10.2 mmol/L.

C. Disorders and pathogeneses (Table 9.14)

1. Inadequate delivery of O_2 to tissues: Hypoxia may occur from recent muscular exercise or disorders causing hypoxemia, hemoglobic (hypemic) hypoxia, or stagnant hypoxia (see Chapter 10). During anaerobic (hypoxic) conditions, less pyruvate and L-lactate enter the Krebs cycle and more L-lactate diffuses from cells (Figure 9.11). This process generates L-lactate, not lactic acid; the formation of L-lactate from glucose occurs with no net gain of H^+ (see Acidemia of lactic acidosis in Chapter 10). However, H^+ accumulates under anaerobic conditions (and other states with insufficient aerobic metabolism to balance glycolysis and energy needs) because the H^+ generated from the hydrolysis of ATP for energy is not consumed by the Krebs cycle and oxidative phosphorylation.

Table 9.14. Disorders and conditions that cause hyperlactatemia (of L-lactate)

Inadequate delivery of O_2 to tissues
Stagnant hypoxia:* cardiogenic or hypovolemic shock, cardiac failure, blood vessel occlusion (e.g., thrombosis, severe trauma)
Demand hypoxia:* strenuous exercise, seizures, struggling during restraint
Hypoxemia: respiratory disorders
Hemoglobic (hypemic) hypoxia: severe anemia, methemoglobinemia, carboxyhemoglobinemia
Increased production by metabolic pathways of the animal
Fear or excitement (fight-or-flight response)
Shock and potentially other critical illness (β_2-adrenergic stimulation)
Exogenous β_2-adrenergic agonists (salbutamol, salmetrol)
Methylxanthine toxicity (chocolate, caffeine)
Defective metabolic pathways for aerobic respiration
Hyperammonemia: urea toxicosis, ammoniated forage toxicosis
Pyruvate dehydrogenase deficiency
Thiamine (vitamin B_1) deficiency
Malignancy, large or widespread with increased aerobic glycolysis (potential, rare)
Increased production by alimentary-tract bacteria: grain overload, ruminal acidosis, forestomach acidosis
Other or unknown mechanisms
Bacterial sepsis
Canine babesiosis
Hepatic disease, severe
Transfusion of stored blood or packed RBCs
Metformin toxicity
Propylene glycol administration or toxicity

* A relatively common disorder, condition, or mechanism

Note: [L-Lactate] is greater in healthy neonatal foals (1–3 d) than in adult horses.

a. Stagnant hypoxia
   (1) Shock: The redistribution of blood results in poor perfusion of tissues, which causes stagnant hypoxia and increased generation of L-lactate. However, a portion of the increased L-lactate production may be from epinephrine-induced stimulation of glycolysis in muscle.[185]
   (2) Cardiac failure and vessel occlusion (e.g., thrombosis, severe trauma) can also impair circulation and oxidative metabolism.
   (3) Equine colic caused by gastrointestinal disorders (e.g., torsion, strangulation, and infarction): The excessive L-lactate production may be due to poor perfusion of tissues, but absorbed endotoxins may also stimulate L-lactate production, and intestinal bacteria may contribute L-lactate (and D-lactate). In one study involving 14 horses with increased AGs, L-lactate concentrations were about 10 times D-lactate concentrations and thus the increased AG was due to increased [L-lactate].[186] Generally, a greater severity of hyperlactatemia corresponds to a poorer prognosis in colicky horses.[187]

b. Demand hypoxia due to strenuous exercise: Excessive muscular activity increases anaerobic respiration and thus increases L-lactate production and release from muscle.[188]

c. Hypoxemia: Poor oxygenation of blood could produce sufficient hypoxia to cause hyperlactatemia, but this situation is not commonly recognized.

d. Hemoglobic (hypemic) hypoxia: Defective transport of O_2 to tissues could produce hyperlactatemia, but this situation is not commonly recognized and probably occurs only with severe anemia, marked methemoglobinemia, or marked carboxyhemoglobinemia.

2. Increased production by metabolic pathways of the animal
   a. Fear or excitement (fight-or-flight response)
      (1) In experimental studies of stress-induced hyperglycemia, cats struggling with harness restraint while being sprayed with water had increased plasma L-lactate concentrations (mean peak concentration = 64 mg/dL; baseline concentrations < 10 mg/dL). The hyperlactatemia was considered to be caused by increased muscular activity (struggling),[189] but increased production from glycolysis secondary to β_2-adrenergic stimulation by catecholamines may have contributed (see next entry regarding shock).[185] Similar changes in other animals would be expected with similar conditions.
      (2) In a study of 21 cats, no difference was found in plasma L-lactate concentrations among cats that struggled while blood was collected via venipuncture versus those that did not struggle or struggled less.[190] Blood was collected from occluded veins, and one definitely higher [L-lactate] was considered an outlier.
   b. Shock and potentially other critical illness (β_2-adrenergic stimulation)
      (1) Although hyperlactatemia in patients with septic shock, hemorrhagic shock, or other critical illness has been considered secondary to hypoxia-induced anaerobic metabolism, evidence supports a role for increased glycolysis and Na^+-K^+-ATPase activity secondary to β_2-adrenergic stimulation.[98,185]
      (2) Catecholamine stimulation of β_2-adrenergic receptors on myocytes promotes glycogenolysis and glycolysis, thus generating L-lactate. It also causes myocytes to increase Na^+-K^+-ATPase activity, thereby increasing consumption of ATP and formation of ADP. The decrease in ATP and increase in ADP stimulates glycolysis (phosphofructokinase), thus generating

more L-lactate. L-Lactate is transported down a concentration gradient to plasma. Increased Na^+-K^+-ATPase activity concurrently promotes hypokalemia by shifting K^+ from ECF to ICF.

c. Exogenous β_2-adrenergic agonists (e.g., salbutamol) – see previous entry for mechanism

d. Methylxanthine toxicity: Theobromine and caffeine (1,3,7-trimethylxanthine) stimulation likely contributes to the hyperlactatemia and hypokalemia reported in many dogs after ingestion of chocolate.[100] Potential mechanisms include (i) secondary cholinergic effects with activation of Na^+-K^+-ATPase pumps and (ii) inhibition of phosphodiesterase, which could enhance or mimic cholinergic effects. Hyperlactatemia and hypokalemia may occur with excessive exposure to other sources of these or other methylxanthines (e.g., theophylline) as reported in people.[191,192]

e. Defective metabolic pathways for aerobic respiration

(1) Hyperammonemia may occur during excessive production of NH_4^+ (e.g., urea toxicosis or ammoniated forage toxicosis), from various causes of hepatic insufficiency, or from a rare urea cycle defect. High [NH_4^+] can interfere with the Krebs cycle such that the aerobic production of ATP is defective. Generation of ATP switches to anaerobic respiration, which produces L-lactate.[193,194]

(2) Pyruvate dehydrogenase deficiency, reported in Sussex spaniels and Clumber spaniels, results in excess formation of L-lactate.[195,196] Pyruvate dehydrogenase catalyzes the conversion of pyruvate to AcCoA, which then enters the Krebs cycle (Figure 9.11). With pyruvate dehydrogenase deficiency, the conversion of pyruvate to L-lactate by LD is increased.

(3) Thiamine (vitamin B_1) deficiency can cause hyperlactatemia because thiamine is an essential cofactor for pyruvate dehydrogenase (see immediately above). Hyperlactatemia may be detected at patient presentation or develop as part of refeeding syndrome.[197,198]

(4) Inhibition of oxidative metabolism by several other substances (e.g., cyanide) could uncommonly cause significant hyperlactatemia in domestic mammals.

f. Although metabolism in malignant cells may be altered to promote aerobic glycolysis and L-lactate generation (Warburg effect), hyperlactatemia in cancer patients is often attributable to other mechanisms.[199]

3. Increased production by alimentary-tract bacteria: When there is excess intake of starch (grain overload in ruminants, horses, ponies, and camelids), there is increased formation of L-lactate and D-lactate by rumen (ruminants), hindgut (equids), or forestomach (camelids) bacteria. Increased lumen acidity alters the microbiome, which may intensify lactate production and diminish lactate use by the microbiota. The lactate is absorbed by the mucosa, thus increasing plasma lactate concentrations. Besides the hyperlactatemia, other findings may include metabolic acidosis, hyperchloremia, hyperglycemia, and features of dehydration (azotemia, hyperalbuminemia).

4. Other or unknown mechanism

a. Bacterial sepsis: The mechanism of hyperlactatemia (L-lactate) is frequently considered to be poor tissue perfusion,[200] but endotoxemia by itself has been reported to cause hyperlactatemia in people.[96] Also, experiments in dogs indicate that the use of L-lactate by liver and other tissues during bacterial sepsis is decreased.[201] Lastly, release of epinephrine during bacterial septic shock increases

aerobic glycolysis and production of L-lactate by muscles or other cells.[185] This might be an important contributor to hyperlactatemia in septic shock.[202]

b. Canine babesiosis: Hyperlactatemia (L-lactate) occurs in some dogs with acute babesiosis, but the pathogenesis of the hyperlactatemia is not established.[203] As for the hyperlactatemia of human malaria, several mechanisms may contribute.[204] It does not appear to be related to the degree of anemia and thus is not caused simply by hemoglobic hypoxia. Given the concurrent hypoglycemia in many dogs, increased glycolysis by host cells may contribute to hyperlactatemia. Increased glycolysis by protozoa might also contribute when numerous organisms are present. Consumption of glucose, mostly by mammalian cells, is a feature of sepsis-induced hypoglycemia (see Glucose Concentration, Section IV.C.1g, in Chapter 14).

c. Hepatic disease: Severe hepatic disease may cause hyperlactatemia (L-lactate), but the pathogenesis is not established. The hyperlactatemia might be due to increased glycolysis and L-lactate production by mostly extrahepatic manifestations of liver disease as well as decreased hepatic utilization of L-lactate for energy or gluconeogenesis.[205]

d. Transfusion of stored blood or packed RBCs: During storage, RBCs continue glycolysis to produce L-lactate, and thus L-lactate-rich blood is administered IV.

e. Metformin toxicity: Metformin is a biguanide compound that is used commonly as an antidiabetic agent in people. One side effect of metformin treatment is hyperlactatemia that probably is due to impaired use of L-lactate (gluconeogenesis) by hepatocytes.[206] Metformin toxicity has occurred in dogs and cats that consumed an owner's metformin medication; hypoglycemia was a concurrent finding (see Hypoglycemia, Section C.2.d in Chapter 14). Experimentally, a dose-response effect of metformin on blood [L-lactate] and pH has been demonstrated in dogs.[207]

f. Propylene glycol administration or toxicity: Propylene glycol is in numerous products and is a rapidly absorbed feed additive and a vehicle for some oral and IV medical products. It is a mixture of D and L stereoisomers, but generally in unknown proportions. A substantial proportion of it is excreted renally, but the majority is metabolized in the liver where alcohol dehydrogenase rapidly converts it to L- and D-lactaldehyde (original stereoisomerism is maintained) with subsequent transformation either to (i) methylglyoxal and then D-lactate by glyoxylases or (ii) L-lactate and D-lactate by aldehyde dehydrogenase, although not all species (e.g., rabbits) can convert D-lactaldehyde to D-lactate by this pathway.[208] Unlike L-lactate, D-lactate is not a good metabolic substrate and is therefore cleared more slowly. L-Hyperlactatemia may resolve quickly, or perhaps not occur with lower doses of propylene glycol, whereas D-hyperlactatemia may develop and persist. Accidental administration or ingestion of excessive amounts of propylene glycol has caused marked hyperlactatemia and clinical signs. Administration of activated charcoal containing propylene glycol to healthy dogs caused hyperlactatemia when used at the high end of the recommended dose.[209]

IV. Increased plasma D-lactate concentrations

A. [D-Lactate] may be increased in animals with diabetes mellitus.[177]

1. D-Lactate concentrations were greater in cats with ketotic diabetes mellitus (mean ≅ 340 μmol/L) than in cats with nonketotic diabetes mellitus (mean ≅ 140 μmol/L) or in healthy cats (mean ≅ 25 μmol/L). D-Lactate may have been formed through a pathway involving acetone or methylglyoxal.

2. Even though there was a significant difference in D-lactate concentrations among the groups, the increase was < 1 mmol/L and thus would not contribute clinically to increased AG values. Also, the D-lactate concentrations are much less than those found in mammals with L-lactate acidosis (e.g., 10–20 mmol/L).

B. Absorption of D-lactate (produced by enteric bacteria) can increase [D-lactate], which is not measured by routine lactate assays but would be contributory to increased AGs when present. Interpretation of published data for [D-lactate] can be challenging because D-lactic acid and D-lactate are sometimes used as synonyms; D-lactate contributes to an AG, but D-lactic acid does not.
   1. D-Lactate is a major contributor to total [lactate] in neonatal calves with diarrhea.[147,210]
   2. In milk-fed calves, failure of esophageal groove reflex allows milk to enter the reticuloruminal cavity (called *ruminal* drinking) in which bacterial fermentation of lactose results in the formation of D-lactic and L-lactic acids or D- and L-lactate. After the ions are absorbed (probably in the intestine), a metabolic acidosis with increased plasma concentrations of D-lactate (near 10 mmol/L)[210] may develop. The rapid metabolism of L-lactate may prevent it from accumulating in the plasma;[211] however, the metabolic clearance rate of D-lactate and L-lactate were shown to be similar in dogs.[212]
   3. A cat with exocrine pancreatic insufficiency had metabolic acidosis and a concurrent increase in plasma [D-lactate] (17.7 mmol/L; 3 healthy cats < 0.3 mmol/L). The authors concluded that the uncommon maldigestive disorder caused enteric production of D-lactate by bacteria.[213] In a study of 100 cats with clinical gastrointestinal disease, the [D-lactate] was 1.0–3.0 mmol/L in 10 and 8.3 mmol/L in 1; 30 healthy cats had [D-lactate] < 0.9 mmol/L.[214]
   4. In a group of 50 colicky horses with mild lactic acidosis, none had increased D-lactate concentrations.[215] In another study of 90 colicky horses, minor increases in plasma [D-lactate] were found and were positively correlated with peritoneal fluid [D-lactate] (see Chapter 19).[216]

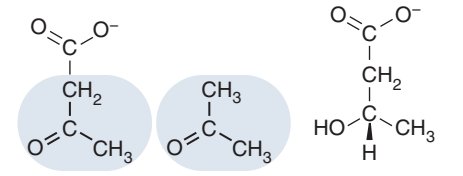
C. Ingestion of propylene glycol may result in increased [D-lactate].[217]
   1. Cats that ingested daily diets of 12 % and 41 % propylene glycol on a dry weight basis had an increased [D-lactate]. The lower percentage, which was near that found in some commercial cat foods at the time, produced nearly a 2 mmol/L increase in [D-lactate], whereas the higher dose produced nearly a 7 mmol/L increase by day 24. Corresponding with the increased [D-lactate], the AGs increased. [L-Lactate] decreased slightly from the small baseline concentrations. The United States Food and Drug Administration banned the use of propylene glycol in cat food.
   2. Propylene glycol is converted to D-lactate via the alcohol dehydrogenase–catalyzed formation of methylglyoxal, which is then converted to D-lactate and glutathione by glyoxylases.[218] In some species (unclear which other than rats and mice), aldehyde dehydrogenase can convert D-lactaldehyde generated from D isomers of propylene glycol to D-lactate.[208]
   3. Published clinical cases of excessive propylene glycol intake in domestic mammals are rare, and laboratory characterization has been the exception.[219] Findings may vary with the dose, timing, and concurrent health status. Expected findings after acute uptake of a large amount of propylene glycol include a high-AG metabolic acidosis with D-hyperlactatemia, hyperosmolality, and an increased osmole gap.[220] D-Lactate may falsely increase [L-lactate] with assays that are not specific for L-lactate, but

[L-lactate] may also be truly increased when it is formed faster than it is metabolized and excreted.

V. Relationship to the AG
   A. Both L-lactate and D-lactate contribute to an AG, when present (see the Anion Gap section). Each 1 mmol/L increase in the [lactate] accounts for 1 mmol/L increase in the AG.
   B. If there is an increased AG in serum or plasma and the [L-lactate] is not increased sufficiently to account for the AG's magnitude, the increased AG could be due to D-lactate or other unmeasured anions (Table 9.12).

β-HYDROXYBUTYRATE (BHB) AND ACETOACETATE (AcAc) CONCENTRATIONS

I. Physiologic processes
   A. Ketogenesis occurs in hepatocytes through a series of reactions that convert AcCoA into BHB, AcAc, and acetone; the two anions (BHB and AcAc) and acetone are collectively called *ketone bodies*, but BHB does not have the chemical structure of a ketone. The amounts of AcAc and BHB in hepatocytes are in equilibrium. The equilibrium shifts toward BHB when NADH is abundant in hepatocytes (e.g., in diabetes mellitus), whereas the equilibrium shifts toward AcAc when NAD^+ is abundant. The pK_a of acetoacetic acid is 3.6, and the pK_a of β-hydroxybutyric acid is 4.7. Therefore, at physiologic pH values, nearly all of these molecules are in their anionic (conjugate base) forms.
   B. Ketogenesis is promoted by glucagon and inhibited by insulin. In most cells (not hepatocytes), AcAc and BHB (after conversion to AcAc) feed into the Krebs cycle and are used for generation of ATP, particularly when glucose is less available.
   C. Renal excretion of BHB helps with the excretion of H^+ because the pK_a of BHB is near that of acidic urine, and thus it binds more H^+ at a urine pH of 5 than at a plasma pH of 7.4. Similarly, AcAc aids in the excretion of H^+ in acidic urine.



ketone bodies (ketone structures are shaded)

   D. Diurnal variations in [BHB] have been reported in cattle. If assessing [BHB] for herd monitoring, standardized collection times may be helpful.

II. Analytical concepts
   A. Units (Note: Conversion factors are for the conjugate base anions and not the corresponding acids.)
      1. AcAc: mg/dL × 99 = μmol/L or mg/dL × 0.099 = mmol/L (SI unit)
      2. BHB: mg/dL × 97 = μmol/L or mg/dL × 0.097 = mmol/L (SI unit)
   B. Assays:
      1. Spectrophotometric quantitative assays use BHB dehydrogenase to measure serum [BHB] by catalyzing the conversion of BHB to AcAc. In some assays, a high [AcAc] can interfere with the reaction to produce a falsely low [BHB].[221] Information was not found

to support and explain lack of AcAc interference as indicated by some manufacturers, but sample dilution can markedly reduce the interference.

2. Electrochemical assays in point-of-care instruments also use BHB dehydrogenase to measure plasma [BHB]; acetone and AcAc do not significantly affect the results.[222–224]
3. The common nitroprusside methods (Ketostix strip, Acetest tablet, and KetoCheck powder) are used with blood, serum, milk, and urine for the detection of AcAc and acetone. BHB does not react because it is not a ketone.[225]
4. Qualitative or semiquantitative methods have been designed to detect BHB in milk; these include the Keto-Test (Ketolac BHB strip).[225,226]

C. Samples: Laboratories may accept serum, anticoagulated whole blood, or plasma from anticoagulated whole blood (EDTA, heparin, oxalate/fluoride), although EDTA and oxalate have been associated with decreased [BHB] in human samples. Hemolysis may alter results when using some assays.

D. BHB stability: BHB is reported to be stable in serum for 1 wk at 4 °C and longer if kept at −20 °C.[227] With bovine samples, results were not affected by a 24 h delay prior to separation of serum or plasma (EDTA or heparin anticoagulation).[228] In human whole blood, [BHB] is stable for 48 h.[229] Other tests on the same sample (e.g., nonesterified fatty acids) may require additional sample-handling considerations.

E. When ketone bodies are detected in blood or serum, they will likely be in urine and, for dairy cows, will often be in milk. However, when comparing results, the type of assay and its analytic properties should be considered. For example, the [BHB] might be increased in serum and urine, but the urine test for ketones (AcAc, perhaps acetone) may not be positive. However, typically the urinary excretion of both BHB and AcAc is sufficient for the nitroprusside method to provide a positive ketone (AcAc) reaction.

F. In theory, BHB can be converted with H_2O_2 to AcAc so that nitroprusside methods can be used to detect BHB. However, BHB concentrations must be > 50 mmol/L in urine or > 100 mmol/L in serum (using 30 % H_2O_2) to produce more than a trace reaction. Such concentrations are not clinically relevant for serum samples, and if urine BHB concentrations were this great, AcAc concentrations would be great enough to be detected routinely (see Chemical Examination of Urine, Section V, in Chapter 8).[230] To put the concentrations in perspective, a 4+ ketone reaction via Multistix suggests the [AcAc] is 160 mg/dL, which is only ~16 mmol/L.

III. Increased ketone body concentration

A. An increased concentration of ketone bodies in blood is *hyperketonemia*, also called *ketonemia*, but the latter does not describe an increase from the measurable concentrations of plasma ketone bodies present in health. The clinical disorder is called *ketosis*; acidemia commonly accompanies ketosis and its pathogenesis is explained in Chapter 10 and Figure 10.8. Ketosis occurs primarily for two reasons:

1. Excessive β-oxidation of fatty acids: This results in the production of more AcCoA than can be used by the Krebs cycle. The excess AcCoA, especially with glucagon excess or insulin deficiency, enters the ketogenic pathway to form AcAc, which can be converted to either acetone or BHB (Figure 10.8). This occurs during diabetes mellitus, starvation, or any negative energy balance that results in excessive β-oxidation of fatty acids.
2. Oxaloacetate deficiency: The AcCoA produced from oxidation of fatty acids typically combines with oxaloacetate as it enters the Krebs cycle, eventually yielding ATP. When

an animal is in a negative energy status, oxaloacetate is used for gluconeogenesis, so there may be an inadequate amount of oxaloacetate to react with AcCoA. This oxaloacetate deficiency is especially important in ruminants, because their major source of plasma glucose is gluconeogenesis. The accumulation of AcCoA promotes ketogenesis.[231] Ketotic cattle may not be acidotic.[232]

B. Conditions and disorders with hyperketonemia
   1. All mammals: starvation, prolonged anorexia, and diabetes mellitus
   2. Cattle: bovine ketosis associated with freshening and the high-energy demands of lactation, displaced abomasum, and hepatic lipidosis
   3. Dogs: lactation and endurance racing
   4. Horses: endurance racing and hyperpituitarism

C. The abilities of cowside tests to detect bovine ketosis can vary.
   1. Three cowside tests were evaluated for detecting bovine ketosis. KetoCheck for AcAc in milk, Ketostix for AcAc in urine, and Keto-Test for BHB in milk.[225] The gold standard in the study was serum [BHB]: Ketosis was considered to be present if the serum [BHB] was > 1.4 mmol/L. The diagnostic properties varied with the decision thresholds, but generally the diagnostic sensitivity and diagnostic specificity of the Ketostix and Keto-Test methods were good. The diagnostic sensitivity of the KetoCheck was considered inadequate for use as a screening test for subclinical ketosis. In a similar study in which hyperketonemia was defined by a whole blood [BHB] > 1.2 mmol/L as assessed by a point-of-care Precision Xtra meter, Ketostix (AcAc in urine) also somewhat outperformed Keto-Test (BHB in milk), with both of these tests registering increases about 2 d after increases in blood [BHB].[233]
   2. Results of a blood-strip test, a milk-strip test, and an automated milk-analyzer test from over 400 cows were compared. Of the 53 cows with increased [BHB], 23 had a positive milk-strip test and 18 had a positive milk-analyzer test.[234]

OSMOLALITY, OSMOLARITY, AND OSMOLE GAP

I. Reasons for measuring serum osmolality and calculation of an osmole gap
   A. There are two primary reasons for measuring serum osmolality:
      1. To determine if osmolality is sufficiently increased to cause a hyperosmolar syndrome
      2. To determine if there is an increased osmole gap by comparing the osmolality of a patient's serum (Osm_m) with its Osm_c, an estimate of osmolality
   B. Neither Osm_m nor Osm_c should be used to determine an animal's hydration status. Dehydration causes an increased osmolality if there is hypernatremic dehydration, but most dehydrated animals are normonatremic or hyponatremic and the Osm_m is WRI or decreased (unless there is increased concentration of another solute such as urea or glucose). In dehydrated animals, the Osm_c is primarily dependent on the animal's $[Na^+]$.

II. Definitions
   A. *Mole*: the SI unit for the amount of substance present when there are 6.022×10^{23} (Avogadro's number) identical particles of it (based on the number of particles in 0.012 kg of carbon 12). One mole of a substance weighs its gram molecular weight (e.g., 1 mole of glucose weighs 180 g and 1 mole of Na^+ weighs 23 g).
   B. *Solute*: a substance dissolved in a solvent

C. *Solvent*: a liquid in which a solute is dissolved to form a solution; the solvent in plasma is H_2O
D. *Osmolality*: the concentration of a solute expressed in moles of solute per *kilogram* of solvent (mol/kg); in the clinical assessment of body fluids, osmolality is expressed as mmol/kg or mOsm/kg (the authors prefer the simpler SI unit of mmol/kg)
E. *Osmolarity*: the concentration of a solute expressed in moles of solute per *liter* of solution (mol/L); in clinical assessments, it is also expressed as mOsm/L (Note: Although similar, serum osmolality does not equal serum osmolarity, because 1 L of normal serum contains about 930 mL of H_2O (0.93 kg of H_2O); in the absence of postprandial lipemia, the remaining volume (~70 mL) is occupied mostly by proteins.)
F. *Osmole*: 1 mole of osmotically active particles, which equals 1 osmole for a substance that does not dissociate in solution; for a substance that completely dissociates into two ions per mole, there are 2 moles of dissociated solute particles per 1 mole of undissociated substance (most dissociable solutes do not dissociate completely)
G. *Osmotic pressure*: the force required to counterbalance the force of osmotic solvent flow through a semipermeable membrane, such as cell membranes; also referred to as *total osmotic pressure* to differentiate it from colloid osmotic pressure (Figure 9.13)
H. *Osmosis*: the passage of solvent (e.g., H_2O) from a solution of lesser solute concentration (greater [H_2O]) through a semipermeable membrane (one that does not permit passage of some solute particles) to a solution of greater solute concentration (lesser [H_2O])
I. *Osmometry*: any technique for measuring the osmolality or osmotic pressure of a solution
J. *Osmometer*: a device or instrument that measures the osmolality (osmotic concentration) or osmotic pressure of a solution; a freezing-point osmometer measures osmolality, and a membrane osmometer measures colloid osmotic pressure
K. *Tonicity*: the *effective* osmolality of a solution, i.e., that solute concentration that can contribute to movement of H_2O across a semipermeable membrane
L. *Colloid osmotic pressure* (oncotic pressure): This is the osmotic pressure exerted by colloidal particles suspended in a solvent. Colloidal particles are macromolecules that are too small (1 nm to 1 μm) to be settled out by gravity. Most of the oncotic pressure of plasma is caused by plasma proteins (about 80 % albumin and 20 % globulins); they create the osmotic pressure gradient in capillaries. Part of colloid osmotic pressure is attributed to cations (e.g., Na^+) that are attracted to the negatively charged proteins (Donnan equilibrium effect). Most nonprotein solutes do not contribute to osmotic pressure in capillaries, because the capillaries are permeable to H_2O and the small solutes. Chapter 7 contains information regarding the measurement and interpretation of colloid osmotic pressures.

III. Physiologic processes
A. As stated in the foregoing definitions, osmolality is the concentration of solutes per kilogram of solvent and therefore depends on the number of molecules or ions in the solution. The major contributors to serum osmolality and their relative contributions are listed in Table 9.15 in terms of their osmolarities (although total osmolality can be measured, the osmolality of each contributing molecule alone can only be estimated from osmolarity).
B. Effective osmolality (tonicity)
1. When different concentrations of solute are on two sides of a membrane that is permeable to H_2O, the solutes establish an osmolal gradient that promotes osmosis (movement of H_2O to diminish the gradient). If the membrane is impermeable to a solute, then that solute contributes to effective osmolality.

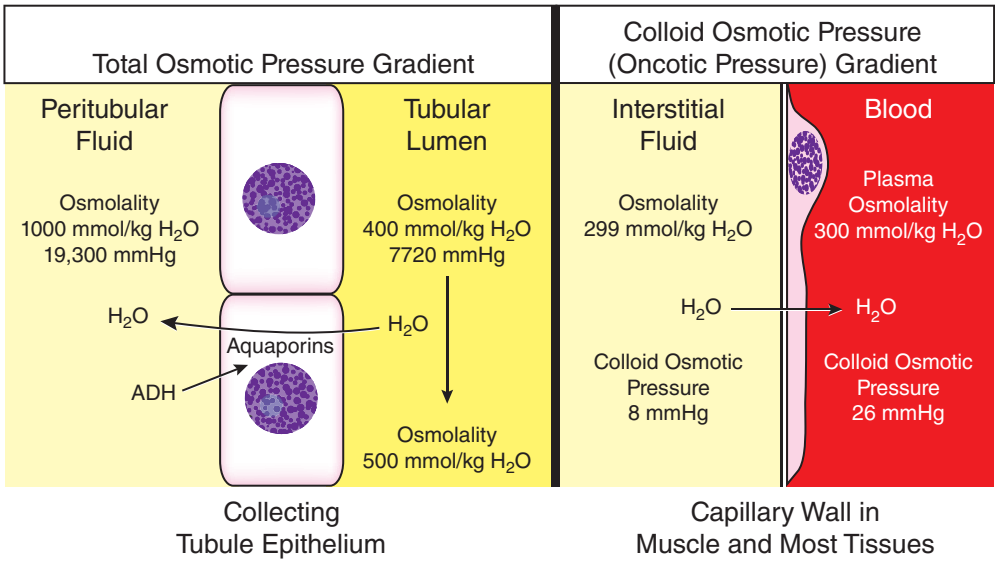


Figure 9.13. Schematic comparison of total osmotic pressure and colloid osmotic pressure.
Left: In the left drawing of the renal collecting tubule under physiologic conditions, the peritubular fluid has a much greater osmolality than the tubular fluid. Osmotic pressure can be calculated from osmolality (each 1 mmol/kg of osmolality is equivalent to 19.3 mmHg of osmotic pressure). Therefore, an osmolality of 1000 mmol/kg equates to an osmotic pressure of 19,300 mmHg, and an osmolality of 400 mmol/kg creates 7720 mmHg of osmotic pressure. The large difference in osmolality creates a large osmotic pressure gradient (almost 12,000 mmHg in this example). The peritubular fluid's hypertonicity is due mostly to high concentrations of Na^+, Cl^-, and urea. When ADH is present (Figure 8.1), it increases the permeability of the tubular epithelium to H_2O (via aquaporins) so that H_2O is resorbed, thus increasing the solute concentration in the tubular fluid.
Right: In the right drawing of a capillary wall in skeletal muscle, the colloid osmotic pressure (synonym: oncotic pressure) is greater in the blood than in the interstitial fluid, both because the protein concentration is greater in plasma than in interstitial fluid and because of the related Gibbs–Donnan equilibrium (see Chapter 7, Colloid Osmotic Pressure). The colloid osmotic pressure gradient (18 mmHg in this example) tends to cause the movement of H_2O (and small solutes) from interstitial fluid to plasma. The movement of H_2O out of plasma is determined by the hydraulic pressure gradient across the semipermeable membrane (see the Colloid Osmotic Pressure section in Chapter 7, and General Concepts and Definitions, Section V, in Chapter 19).

Note: The pressure values are provided to illustrate the magnitude of the difference between the forces of renal osmotic pressure gradients and colloid osmotic pressure gradients in most capillaries. In the renal medulla, osmotic pressure gradients across tubules are > 600 times the colloid osmotic pressure gradient across capillaries.

2. Urea is a small (M_r = 60), polar, and therefore hydrophilic molecule with a neutral charge. Cell membranes have low permeability to urea, but simple diffusion leads to equilibration of urea concentrations across most cell membranes. Some cells (e.g., RBCs, hepatocytes, certain renal tubular epithelial cells) have membrane facilitative urea transporters that are necessary for more rapid movement of urea. In most tissues, intracellular and extracellular urea concentrations have equilibrated, so, unless there is an early, rapid change in urea concentration, urea does not contribute to an osmolal gradient and thus does not contribute to the effective osmolality. This property of urea can be compared with properties of other solutes.

Table 9.15. Analyte-associated contributions to plasma or serum osmolarity in health (approximate concentrations provided to simplify concept)

Analyte	Measured concentration	Contribution to osmolarity[a]	Contribution to total osmolarity in serum of healthy animal
Na^+	146 mmol/L	146 mmol/L	281 mmol/L 95 % of total solute
K^+	4 mmol/L	4 mmol/L	
Cl^-	107 mmol/L	107 mmol/L	
HCO_3^-	24 mmol/L	24 mmol/L	
UN	20 mg/dL[b]	7 mmol/L	12 mmol/L 4 % of total solute
Glucose	100 mg/dL[b]	5.5 mmol/L	
Pi[c]	4 mg/dL[b]	1.3 mmol/L	~3 mmol/L 1 % of total solute
tCa^{2+} [d]	10 mg/dL[b]	~1.0 mmol/L	
tMg^{2+} [d]	1 mmol/L	< 0.5 mmol/L	
Protein	7 g/dL	< 1.0 mmol/L	No significant contribution
Total		≅ 297 mmol/L	

[a] The provided contributions of ionic analytes would be true if each were present only as free ions, but some are linked by ionic bonds (e.g., Na^+/ Cl^-, Na^+/HCO_3^-, Ca^{2+}/PO_4), with each complex contributing the same to osmolarity as each single free ion. Contributions of UN and Pi reflect urea and PO_4, respectively.

[b] See Table 1.6 for converting mg/dL to mmol/L.

[c] A measured Pi of 4 mg/dL would represent about 12.3 mg/dL of PO_4, which would contribute about 1.3 mmol/L to osmolarity if all the PO_4 anions existed as free anions.

[d] The contribution of tCa^{2+} and tMg^{2+} to osmolarity is through their free forms, which are present at approximately 50 % of their total concentrations.

a. Methanol (M_r = 32), ethanol (M_r = 46), and ethylene glycol (M_r = 62) are also small polar molecules that cross cell membranes and are considered ineffective osmoles; thus, they increase osmolality without significantly changing H_2O distribution or $[Na^+]$.
b. Mannitol (M_r = 182), glycerol (M_r = 92), sorbitol (M_r = 182), and glucose (M_r = 180) do contribute to effective osmolality because they are not freely diffusible across most cell membranes.

3. If urea is contributing to an increased serum osmolality and other data do not suggest the presence of other ineffective solutes (e.g., ethylene glycol), effective osmolality of serum or plasma may be estimated (Eq. 9.7) from an osmolality measurement (not commonly done).

$$\begin{aligned}\text{effective osmolality} &= \text{measured osmolality} - \frac{[\text{UN}]}{2.8} \quad \textit{if } [UN] \textit{ is in } mg/dL \\ &= \text{measured osmolality} - [\text{urea}] \quad \textit{for } [urea] \textit{ in } mmol/L\end{aligned} \tag{9.7}$$

4. Knowledge of effective osmolality is useful in two clinical situations:
   a. Determining the presence of a hyperosmolar (hyperosmolal) syndrome
      (1) When the effective plasma osmolality rapidly rises (e.g., to > 350 mmol/kg, $[Na^+]$ > 170 mmol/L, or [glucose] > 600 mg/dL),[235] H_2O leaves cells, including neurons. Consequently, the animal can be depressed, stuporous, or even die from the hyperosmolal state.

(2) However, if the hyperosmolal state develops slowly, then intracellular production of organic molecules such as taurine, glycine, glutamine, sorbitol, and inositol (so-called idiogenic osmoles) diminishes the osmotic gradient, and thus less H_2O leaves the cells. If hypotonic fluids are then administered to reduce the plasma osmolality, there can be a rapid influx of H_2O into neurons and development of cerebral edema.

b. Determining physiologic responses that are expected in azotemic animals

(1) If hyperosmolality is caused only by increased urea concentrations (i.e., effective osmolality is not increased), there should not be a stimulus to drink H_2O or release ADH (unless hypovolemia is present).

(2) If hyperosmolality is due to effective solutes (e.g., ethylene glycol), the hyperosmolality is expected to stimulate thirst and ADH release.

C. Major concepts regarding the relative contributions of solutes to serum osmolality

1. Na^+ is the major solute in serum. About half of the solute (moles, not mass) in serum is actually Na^+ ions.
2. Cl^- runs a close second. Because Cl^- frequently follows Na^+, changes in $[Na^+]$ are frequently accompanied by changes in $[Cl^-]$, which approximately doubles the changes in osmolality by Na^+ alone.
3. At physiologic concentrations, urea and glucose are small contributors to total osmolality. However, marked azotemia or hyperglycemia cause hyperosmolality.
4. There are relatively minor differences in urea and glucose concentrations among healthy mammals, and thus most of the differences in serum osmolality are due to differences in Na^+ and Cl^- concentrations.
5. Protein contributes a relatively large amount of mass to plasma solids but very little to osmolality (< 1 mmol/kg). Protein molecules are relatively very large but extremely rare compared to electrolyte ions and urea and glucose molecules.

D. Hypothalamic osmoreceptors are sensitive to increases and decreases in effective plasma osmolality (tonicity).

1. If effective osmolality is increased, ADH is released to stimulate the renal collecting ducts to resorb H_2O. In addition, the thirst center is stimulated to increase the intake of H_2O. Both processes cause a dilutional correction of the increased plasma solute concentration.
2. If the effective osmolality is decreased, ADH secretion is diminished, and thus there is less H_2O resorption by the collecting ducts, increased H_2O loss, and thus a correctional increase in concentration of plasma solutes.

IV. Analytical concepts for osmolality and osmolarity

A. Osm_m

1. Osmometry is based on physiochemical properties (called *colligative properties*) that depend on the number, not the type, of particles in a solution. Two colligative properties are used in clinical osmometry:

a. Freezing-point depression: A 1 mol/kg solution of a solute in H_2O (1 molal) has a freezing point 1.86 °C lower than that of pure H_2O. This is the basis for salting icy sidewalks or roads; increased solute concentration lowers the freezing point and melts the ice.

b. Vapor pressure (or dew point) depression: A 1 mol/kg solution of a solute in H_2O (1 molal) has a vapor pressure 0.03 mmHg lower than that of pure H_2O.

2. Freezing-point osmometers are more common, more precise, and more accurate than vapor pressure osmometers. Vapor pressure osmometers do not measure the contribution of volatile solutes (e.g., alcohols) to total osmolality. (Note: Colloid osmometers measure colloid osmotic pressure and not osmolality; see the Colloid Osmotic Pressure section in Chapter 7.)
3. Osm_m indicates the total concentration of solutes in the serum but does not indicate which solutes are present.
4. Serum is the required sample for measuring osmolality; anticoagulants for plasma samples would add solute to the sample, and therefore increase osmolality. The stability of a serum osmolality depends on the stability of the individual solutes in the sample (see sections for Na^+, K^+, Cl^-, HCO_3^-, glucose, urea, or other solutes that can be present).

B. Osm_c
1. Although some groups recommend the term "calculated osmolality," and it is sometimes reported as "osmolality" (with units of osmolality), Osm_c is calculated from osmolarity values with units of osmolarity, and there is no unit conversion built into the calculation to justify switching from units of osmolarity to units of osmolality. However, the value is an estimate of osmolality. As described in this section, the reported value depends only on the molar concentrations of the three solutes used in the calculation: [Na^+], [urea], and [glucose]. Thus, the Osm_c increases with hypernatremia, azotemia, or hyperglycemia. Significant decreases in the calculated value occur only with hyponatremia.
2. Because there is a direct correlation between molality and osmolality and there is a relationship between molality and molarity, knowing the millimolar concentrations of solutes enables estimation of the osmolality that is expected due to those solutes. However, this conversion is not exact because of three factors:
   a. Osmolality and osmolarity of plasma solutes are not equal because 1 L of plasma contains about 0.93 kg H_2O and about 0.07 kg solids in health. The H_2O percentage will be different with hyperproteinemia, hypoproteinemia, or lipemia.
   b. The concentrations of some plasma solutes are not routinely measured, and their contributions to total osmolality are not included in calculations.
   c. Some plasma solutes are not completely dissociated. The osmotic coefficient of NaCl is 0.93; thus, 1 mmol of NaCl in plasma H_2O contributes 1.86 mmol of active particles (2 × 0.93). There also is an association of Na^+ and HCO_3^- ions in addition to the free HCO_3^- ions. About half of the PO_4 ions are bound to albumin or cations such as Ca^{2+} and Mg^{2+}, and only about half of Ca^{2+} is present as free ions.
3. At least 14 formulas have been used to calculate Osm_c of serum or plasma;[236,237] the most common formulas used in veterinary medicine are shown in Eq. 9.8. Each formula includes measured values for some of the major solutes contributing to osmolarity, and constants or factors to estimate the effects of the other solutes. The formulas vary because of the following:
   a. Different investigators attempted to estimate osmolality by using different solutes and conversion factors.
   b. Method variations for some of the analytes produce different results, which require different mathematical manipulations to best estimate osmolality.
   c. The dissociation of ionic compounds may vary among species.

In the following Eq. 9.8 formulas, Na^+ and K^+ concentrations are in mmol/L or mEq/L. If UN and glucose concentrations are in mg/dL, then:

$$Osm_c = 1.86\ ([Na^+] + [K^+]) + \frac{[UN]}{2.8} + \frac{[glucose]}{18} \quad (\mathbf{9.8a})$$

$$Osm_c = 2\ [Na^+] + \frac{[UN]}{3} + \frac{[glucose]}{20} \quad (\mathbf{9.8b})$$

If urea and glucose concentrations are in mmol/L, then:

$$Osm_c = 1.86\ ([Na^+] + [K^+]) + [urea] + [glucose] \quad (\mathbf{9.8c})$$

$$Osm_c = 2\ [Na^+] + [urea] + [glucose] \quad (\mathbf{9.8d})$$

4. What are the components of the equations?
   a. $1.86 \times ([Na^+] + [K^+])$ or $2 \times [Na^+]$: an estimate of the osmolarity (and osmolality) due to electrolytes, mostly the major four electrolytes, i.e., the sum of Na^+, K^+, Cl^-, and HCO_3^- concentrations.
      (1) Because plasma must remain electrically neutral, increased concentrations of unmeasured anions will be associated with lower $[Cl^-]$ or $[HCO_3^-]$ if cation concentrations remain constant. Thus, the expression estimates the osmolarity due to electrolytes even when there is an increased AG.
      (2) The inclusion of K^+ tends to yield a more accurate Osm_c (estimated osmolality), especially if there is hyponatremia and hyperkalemia. However, because serum $[K^+]$ cannot change much (± 2 mmol/L) without serious medical consequences, clinically major changes in serum $[K^+]$ cause only minor changes in total serum osmolarity.
      (3) When considering the Osm_c, it is important to recognize that the first part of the formula (that which contains Na^+ or $Na^+ + K^+$) represents the contributions of all electrolytes, not just Na^+ or just Na^+ and K^+.
   b. [UN] ÷ 2.8: conversion of [UN] from mg/dL to mmol/L of urea (M_r of UN = 28, and there are 10 dL in 1 L) (a conversion to osmolarity, not osmolality)
   c. [UN] ÷ 3: approximate conversion of [UN] from mg/dL to mmol/L of urea
   d. [Glucose] ÷ 18: conversion of [glucose] from mg/dL to mmol/L (M_r of glucose = 180, and there are 10 dL in 1 L) (a conversion to osmolarity, not osmolality)
   e. [Glucose] ÷ 20: approximate conversion of [glucose] from mg/dL to mmol/L
   f. The conversions of UN and glucose do not include factors to account for plasma containing about 7 % solids, and therefore the UN and glucose values are measures of osmolarity, not osmolality.
5. Osm_c is of little value by itself. If $[Na^+]$, [urea], or [glucose] is increased, then Osm_c will be increased by a similar amount (in mmol/L); however, the Osm_c may or may not estimate serum osmolality. If the Osm_c is not increased, nonmeasured solutes could still be present to create a hyperosmolal state. By itself, an increased Osm_c indicates only that the concentration of one or more of the following is increased: Na^+, urea, or glucose.

C. Osmole gap
1. There is not a correct unit for the osmole gap because the Osm_m is expressed as mmol/kg (molality) whereas the calculation for Osm_c includes measured solute volume concentrations (mmol/L or mg/dL) and is best expressed as mmol/L (osmolarity). Since neither "osmolar gap" nor "osmolal gap" is a correct term, the term *osmole gap* is used. If a unit must be used, perhaps it should be mmol/kg-L to show the mixture of units.
2. Osmole gap formula (Eq. 9.9)

$$\text{Osmole gap} = Osm_m - Osm_c \quad (\mathbf{9.9})$$

a. Osm_m represents the measured total solute concentration (osmolality) in the sample. Osm_c estimates the solute concentration by use of some combination of Na^+, K^+, urea, and glucose concentrations; it is an estimate of osmolarity, and it approximates osmolality.
b. Published osmole gap values should be used cautiously considering the amount of disagreement in laboratory RIs for most solutes (including electrolytes), differences in Osm_c formulas, and potential differences in osmometer results.

V. Evaluation of serum osmolality and osmole gap: The major concepts are shown in Figure 9.14. Identifying an increased osmole gap may be useful to identify the presence of an abnormal nonionic solute (e.g., ethylene glycol or mannitol) (Figure 9.14.E).

VI. Interpretation of osmolality and osmole gap data (Table 9.16)
A. Serum hyperosmolality and osmole gap WRI: An increased Osm_m indicates that the concentration of one or more solutes is increased. Since the osmole gap is WRI, there is a proportionate increase in Osm_c and Osm_m. Because an increased Osm_c results from increased concentrations of Na^+ (and associated electrolytes), urea, or glucose, then the increased Osm_m is due to increased Na^+ (and associated electrolytes), urea, or glucose concentrations.
B. Serum hyperosmolality and increased osmole gap: An increased Osm_m indicates that the concentration of one or more solutes is increased. Because the osmole gap is increased, the Osm_c is not increased or not increased as much as the increase in Osm_m. In this situation, substantial increases in the osmole gap are associated with poisonings by nonelectrolytes (e.g., ethylene glycol, methanol, or paraldehyde) or IV administration of mannitol or radiographic contrast media. The increased osmole gap is due to a nonionic solute other than urea or glucose for the following reasons:
1. Increases in $[Na^+]$, $[K^+]$, or anions (Cl^-, HCO_3^-, or others) would result in increased Osm_m and the same increase in the Osm_c. The Osm_m increases because there are more particles in solution. The Osm_c increases because $1.86 \times ([Na^+] + [K^+])$ (Eq. 9.8a) and $2 \times [Na^+]$ (Eq. 9.8b) estimate the concentration of all electrolytes, assuming that the equations provide reliable estimates of the serum osmolarity. Because the expressions do not account for biological variation in ion concentrations (within an animal, between animals, or among species), they provide only estimates.
2. With an increase in unmeasured anions and the requirement of electroneutrality, there will be either a concomitant increase in cations or a concomitant decrease in other anions.

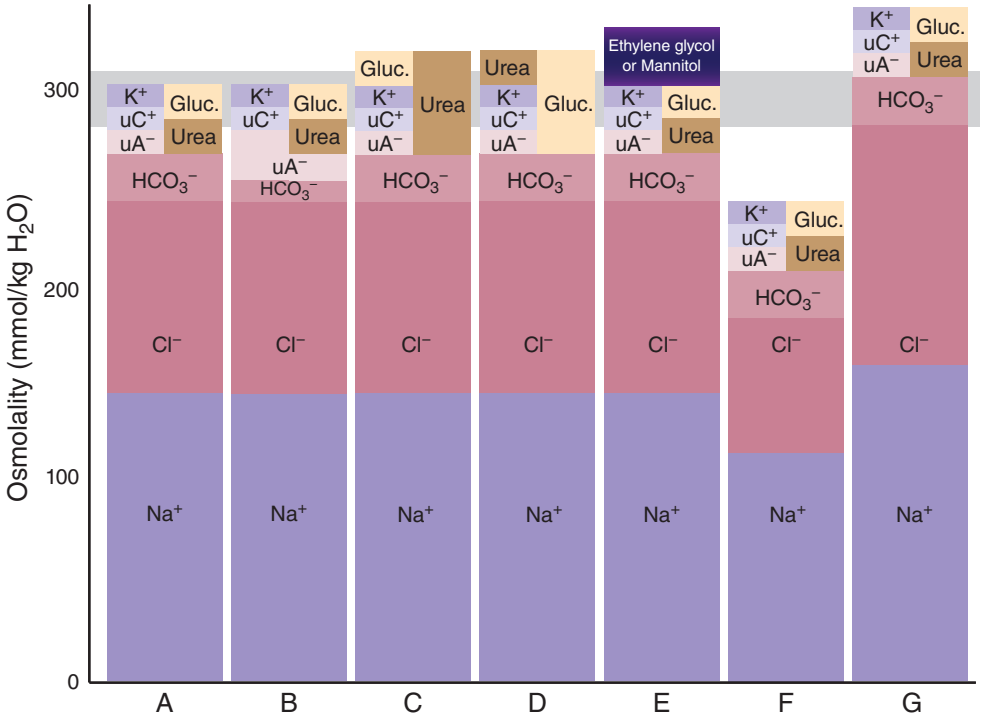


Figure 9.14. Bar diagram of serum osmolality (Osm_m) concepts.
Cations are shades of lavender; anions are shades of mauve. The unmeasured cations (uC^+) are mostly fCa^{2+} and fMg^{2+}, whereas the unmeasured anions (uA^-) are mostly PO_4, SO_4, and anions of organic acids. Proteins are not included because they do not contribute significantly to osmolality. The gray horizontal bar represents a RI for Osm_m; its values vary among species. Osmole gaps are not shown, but are all WRI except for example E.

A. Healthy animals: Nearly all nonprotein solutes are monovalent electrolytes except for urea and glucose (Gluc.), which are uncharged. The osmole gap should be near 0 if an optimized formula for Osm_c is used (one with very good agreement between Osm_m and Osm_c).
B. High-AG, normochloremic, metabolic acidosis (therefore low $[HCO_3^-]$): The osmole gap is not increased because the osmolality is unchanged, and the Osm_c is unchanged because the 1.86 ($Na^+ + K^+$) estimates the contribution of all cations and anions; electroneutrality must be maintained, so increases in the $[uA^-]$ must be accompanied by either increases in cations, decreases in other anions (as in this example with low $[HCO_3^-]$), or both.
C. Hyperosmolality caused by azotemia: The osmole gap is not increased, because both the Osm_m and the Osm_c are increased proportionately by the greater [urea].
D. Hyperosmolality caused by hyperglycemia: The osmole gap is not increased, because both the Osm_m and the Osm_c are increased proportionately by the higher [glucose].
E. Hyperosmolality caused by an abnormal nonionic solute (e.g., ethylene glycol, propylene glycol, or mannitol): The osmole gap is increased by the concentration of abnormal solute (*purple region*), because the exogenous solute increases the Osm_m but is not included in the Osm_c formula.
F. Hypoosmolality caused by hyponatremia and hypochloremia: The osmole gap is not changed because the Osm_m and the Osm_c are decreased by the same amount.
G. Hyperosmolality caused by hypernatremia and hyperchloremia: The osmole gap is not changed because the Osm_m and the Osm_c are increased by the same amount. This hyperosmolal state is the only hyperosmolality that is directly caused by dehydration. Normonatremic and hyponatremic dehydration do not cause hyperosmolality.

Table 9.16. Disorders and conditions that cause abnormal serum osmolality or increased osmole gaps

Osm_m	Osmole gap	Abnormal [solute]	Disorders, conditions	Figure
WRI	WRI[a]	All solutes WRI	Healthy	9.14A
WRI	WRI	↑ uA^-, ↓ HCO_3^-	Metabolic acidoses with ↑ AG	9.14B
↑	WRI	↑ [Na^+]*	↑ [Na^+] (Table 9.2)	9.14G
↑	WRI	↑ [urea]*	↑ [urea] (Table 8.6)	9.14C
↑	WRI	↑ [glucose]*	↑ [glucose] (Table 14.4)	9.14D
↑	↑	↑ concentration of a nonanionic compound other than urea or glucose	Mannitol infusion (IV) Radiographic contrast media (IV) Ethanol, methanol, ethylene glycol	9.14E — 9.14E
↓	WRI	Indicates hyponatremia*	↓ [Na^+]	9.14F
WRI	↑	Pseudohyponatremia	See Sodium concentration, V.B.7.	—

* A relatively common disorder, condition, or mechanism

[a] The RI for osmole gap varies among species and with variations in laboratory assays and formulas used to determine Osm_c.

a. If cations (Na^+ and/or K^+) increase to the same degree that the unmeasured anions increase, the Osm_c will be increased along with Osm_m, so the osmole gap will not be increased.
b. If other anions (Cl^- and/or HCO_3^-) decrease to the same degree that the unmeasured anions increase, the Osm_m and Osm_c will remain unchanged.
c. Either way, and because of electroneutrality, the osmole gap should not increase with an increased concentration of anionic compounds in circulation.

3. The 1.86 ([Na^+] + [K^+]) and 2 × [Na^+] expressions would underestimate the total ion concentration if there were increases in cation concentrations other than [Na^+] or [K^+]. However, increases in [fCa^{2+}] or [fMg^{2+}] of 2–3 mmol/L would create pathologic states but increase the serum osmolality by no more than 4–6 mmol/kg. (The increase in cation charge concentration could be matched by an increase in anion charge concentration, or by a reduction in [Na^+] and/or [K^+].) A change in osmole gap of 4–6 units would typically be considered clinically insignificant because it could represent physiologic or analytical variation.
4. Even though the osmole gap will increase because of an increased concentration of a nonionic solute, there can be a concurrent increase in anions to create an AG. Methanol can contribute to an osmole gap, and its metabolite (formate) can contribute to an AG. Ethylene glycol can contribute to an osmole gap, and its metabolites (glycolate or oxalate) can contribute to an AG. In ketoacidosis, acetone might contribute to an osmole gap, but AcAc and BHB contribute to the AG.

C. Serum hypoosmolality and osmole gap WRI: A decreased Osm_m indicates that the concentration of one or more solutes is decreased. Because the osmole gap is WRI, there is a proportionate decrease in Osm_c and Osm_m, and thus there must be decreased

concentrations of Na^+ (and associated anions), UN, or glucose. Because UN and glucose contribute about 7 and 5 mmol/kg, respectively, in health, and a marked decrease in either (e.g., from 7 to 3 or 5 to 2 mmol/kg) causes only a minor decrease in total osmolality, any clinically significant hypoosmolality is caused by hyponatremia.

D. With pseudohyponatremia (and pseudohypochloremia), the Osm_m is an accurate value because lipids or proteins do not affect the concentration of major osmoles in plasma H_2O. However, the Osm_c may be falsely decreased because of a spuriously low $[Na^+]$. Therefore, the osmole gap will be artifactually increased.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 10

BLOOD GASES, BLOOD pH, AND STRONG-ION DIFFERENCE

Steven L. Stockham and Michael A. Scott

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Table 10.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
$\Delta AG{:}\Delta HCO_3^-$	Delta anion gap to delta bicarbonate ratio
Δ gap	Delta gap
2,3-DPG	2,3-Diphosphoglycerate
AcAc	Acetoacetate
AG	Anion gap
A_{TOT}	Sum or total of nonvolatile weak acids
BE(B)	Base excess in blood
$BE_{(ecf)}$	Base excess in extracellular fluid
BE(P)	Base excess in plasma
BHB	β-Hydroxybutyrate
CO	Carbon monoxide
$CO_{2(g)}$	Gaseous carbon dioxide
CO_3^{2-}	Carbonate
COHgb	Carboxyhemoglobin
$DO_2(Aa)$	Alveolar arterial oxygen tension gradient
ECF	Extracellular fluid
fCa^{2+}	Free ionized calcium
fMg^{2+}	Free ionized magnesium
FO_2Hgb	Fraction of oxyhemoglobin in total hemoglobin
H_2CO_3	Carbonic acid
H_2O	Water
$H_2PO_4^-$	Dihydrogen phosphate
HCO_3^-	Bicarbonate
Hct	Hematocrit
Hgb	Hemoglobin
HHgb	Deoxyhemoglobin (reduced hemoglobin)
HPO_4^{2-}	Hydrogen phosphate
MetHgb	Methemoglobin
O_2Hgb	Oxyhemoglobin
O_2ct	Oxygen content
pCO_2	Partial pressure of carbon dioxide
$pCO_2(a)$	Partial pressure of carbon dioxide in arterial blood
$pCO_2(A)$	Partial pressure of alveolar carbon dioxide
$pCO_2(v)$	Partial pressure of carbon dioxide in venous blood
pH	$-\log [H^+]$
pO_2	Partial pressure of oxygen
$pO_2(a)$	Partial pressure of oxygen in arterial blood
$pO_2(A)$	Partial pressure of alveolar oxygen
$pO_2(I)$	Partial pressure of inspired oxygen

Table 10.1. Abbreviations and symbols in this chapter (Continued)

$pO_2(v)$	Partial pressure of oxygen in venous blood
PCT	Proximal convoluted tubule
PO_4	Phosphate including PO_4^{3-}, HPO_4^{2-}, or $H_2PO_4^-$
PO_4^{3-}	Phosphate
RBC	Red blood cell (erythrocyte)
RI	Reference interval
SID	Strong-ion difference
$SID_{adjusted\ [Cl^-]}$	Strong-ion difference using [Na^+]-adjusted chloride concentration
SID_{true}	True strong-ion difference
SID_x	Strong-ion difference calculated using *x* strong ions
sO_2	Percent hemoglobin saturation with oxygen
$sO_2(a)$	Percent hemoglobin saturation with oxygen in arterial blood
pO_2	Percent hemoglobin saturation with oxygen of arterial blood by pulse oximetry ("p" is for "pulse" oximetry)
tCO_2	Total carbon dioxide
uSA	Unidentified strong anion
WRI	Within reference interval

Note: Blood gas symbols used in this book are those used by the Clinical and Laboratory Standards Institute.[1] Symbols for quantity include partial pressure (*p*), saturation (*s*), and substance fraction (*F*); however, concentrations are denoted by brackets rather than the recommended *c* because of potential confusion with c referring to canine, e.g., in the context of cPLI. Specimen type and source symbols: B, blood; ecf, extracellular fluid; a, arterial; A, alveolar; v, venous. These symbols are combined as appropriate, thus the arterial partial pressure of CO_2 is designated $pCO_2(a)$ and the base excess in extracellular fluid is $BE_{(ecf)}$.

DEFINITIONS

As shown below, acid-base disorders have been defined in different ways. The traditional and buffer-excess definitions are centered on [HCO_3^-]. The SID definition involves use of strong ions (cations and anions) whose activities are considered bases and acids, respectively. This chapter primarily uses the traditional method to explore acid-base disorders. Base-excess calculations are provided, and an overview of the SID method is located at the end of the chapter.

Definitions of important terms in this chapter:

- *Acidemia* is a decreased blood pH (increased [H^+]).
- *Alkalemia* is an increased blood pH (decreased [H^+]).
- *Acidosis* is a condition that produces or promotes a lower blood pH (either toward an acidemia or to reduce an alkalemia as a compensatory change).
  - *Metabolic acidosis* is a condition that decreases the [HCO_3^-] and lowers blood pH (traditional definition using Henderson–Hasselbalch equation).
  - *Metabolic acidosis* is a condition that produces or promotes a decreased strong-ion difference (SID definition).

- *Metabolic acidosis* is a condition that produces or promotes a decreased (more negative) base-excess value (buffer-excess definition).
- *Respiratory acidosis* is a condition that increases the plasma pCO_2 and lowers blood pH (traditional definition using Henderson–Hasselbalch equation).

- *Alkalosis* is a condition that produces or promotes a higher blood pH (either toward an alkalemia or to reduce an acidemia as a compensatory change).
  - *Metabolic alkalosis* is a condition that increases the $[HCO_3^-]$ and raises blood pH (traditional definition using Henderson–Hasselbalch equation).
  - *Metabolic alkalosis* is a condition that produces or promotes an increased strong-ion difference (SID definition).
  - *Metabolic alkalosis* is a condition that produces or promotes an increased (more positive) base-excess value (buffer-excess definition).
  - *Respiratory alkalosis* is a condition that decreases the plasma pCO_2 and raises blood pH (traditional definition using Henderson–Hasselbalch equation).
- *Hypercapnia* is excess CO_2 in blood; it is best recognized as increased pCO_2(a) and perhaps as increased pCO_2(v) (see Analytical Concepts). It is also called *hypercarbia*.
- *Hypocapnia* is a deficiency of CO_2 in blood; it is best recognized as decreased pCO_2(a) and perhaps as decreased pCO_2(v) (see Analytical Concepts). It is also called *hypocarbia*.
- *Hypoxemia* is a deficiency of dissolved O_2 in blood, i.e., decreased pO_2(a).
- *Hypoxia* is a deficiency of O_2 reaching the tissues, cells, or organelles of the body.
- *Hyperventilation* is an excessive rate and/or depth of respiration leading to increased loss of CO_2 from the blood because of increased movement of new air to (and from) the gas-exchange regions of the lung. Because hypocapnia can be the consequence of hyperventilation, sometimes hypocapnia and hyperventilation are considered synonyms.
- *Hypoventilation* is deficient ventilation of the lungs that causes a reduction in the O_2 content of the blood, an increase in the CO_2 content of the blood, or both, because of decreased movement of new air to (and from) the gas-exchange regions of the lung. Because hypercapnia can be the consequence of hypoventilation, sometimes hypercapnia and hypoventilation are considered synonyms.
- *Tachypnea* is an increased rate of respiration. The increased rate may or may not cause hyperventilation.
- *Bradypnea* is a decreased rate of respiration. The decreased rate may or may not cause hypoventilation.

Note: The basis for blood gas symbols is provided in the Table 10.1 footnote. Variations in blood gas notation used by institutions and individuals can be confusing.

PHYSIOLOGIC PROCESSES

I. Several respiratory and nonrespiratory processes help maintain plasma $[H^+]$ at a minute, but stable, concentration (about 40 nmol/L). Metabolic processes continually produce H^+, and it is either excreted (via kidneys) or bound to buffers (HCO_3^-, PO_4, ammonia, sulfates, Hgb, and other proteins, such as albumin). Of the total buffering capacity in health, HCO_3^- contributes over 20 mmol/L in most species, whereas the nonbicarbonate buffers contribute less than 10 mmol/L.[2]

II. As the buffers work together, changes in one buffering system reflect changes in the others. In clinical medicine, the bicarbonate buffering system is used to monitor control of $[H^+]$ and therefore acid-base status.

A. The relationships among $[H^+]$, $[HCO_3^-]$, and pCO_2 in blood at 37 °C can be expressed with the Henderson–Hasselbalch equation or a nonlogarithmic version (Eq. 10.1a). The $[H_2CO_3]$ is calculated from a measured pCO_2 value and the solubility coefficient of CO_2 in an aqueous solution (Eq. 10.1b).

$$pH = 6.1 + \log \frac{[HCO_3^-]}{[H_2CO_3]} \quad \text{or} \quad [H^+] = 24 \times \frac{pCO_2}{[HCO_3^-]} \qquad (\mathbf{10.1a})$$

$$pCO_2 \times 0.0307 = [H_2CO_3] \qquad (\mathbf{10.1b})$$

$[H^+]$ in nmol/L; $[HCO_3^-]$ and $[H_2CO_3]$ in mmol/L; pCO_2 in mmHg

B. As seen in the nonlogarithmic Eq. 10.1a, $[H^+]$ is clearly related to the ratio of pCO_2 to $[HCO_3^-]$. When the ratio increases in an animal, the blood pH shifts toward acidemia, and the animal may become acidemic. When the ratio decreases in an animal, the blood pH shifts toward alkalemia, and the animal may become alkalemic.

III. Pulmonary functions related to blood gases and acid-base status

A. Expiration of CO_2 leads to the elimination of free H^+ from plasma (Figure 10.1).

1. Because blood $[H^+]$ is very low compared to $[HCO_3^-]$ (ratio ≅ 1:600,000), the elimination of H^+ does not lower $[HCO_3^-]$ significantly. If there is excessive generation of H^+ or impaired renal excretion of H^+ during a metabolic acidosis, then $[HCO_3^-]$ decreases when HCO_3^- is used to buffer H^+ and the formed CO_2 is exhaled.

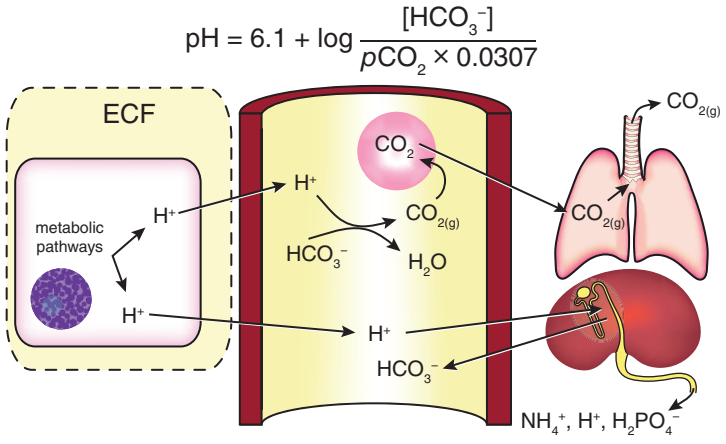


Figure 10.1. Acid-base basics. Metabolic pathways in cells continually produce H^+, which is buffered by HCO_3^-, Hgb, or other buffers so that very little remains as free H^+. After combining with HCO_3^- (in extracellular fluid mostly) and dissociating to form CO_2 and H_2O, the dissolved $CO_{2(g)}$ in plasma creates the pCO_2, and some CO_2 enters RBCs for transportation to lungs (Figure 10.3). When blood passes through the lungs, the $CO_{2(g)}$ is exhaled. The other major route of removing H^+ from blood is via the kidneys. Most renal excretion of H^+ is within NH_4^+ or $H_2PO_4^-$, but a portion is excreted as free H^+. The ability of these processes to maintain physiologic values can be monitored by measuring plasma $[HCO_3^-]$, pCO_2, and pH in a blood sample. The relationship of these analytes is expressed by the Henderson–Hasselbalch equation (in figure and in Eq. 10.1a).

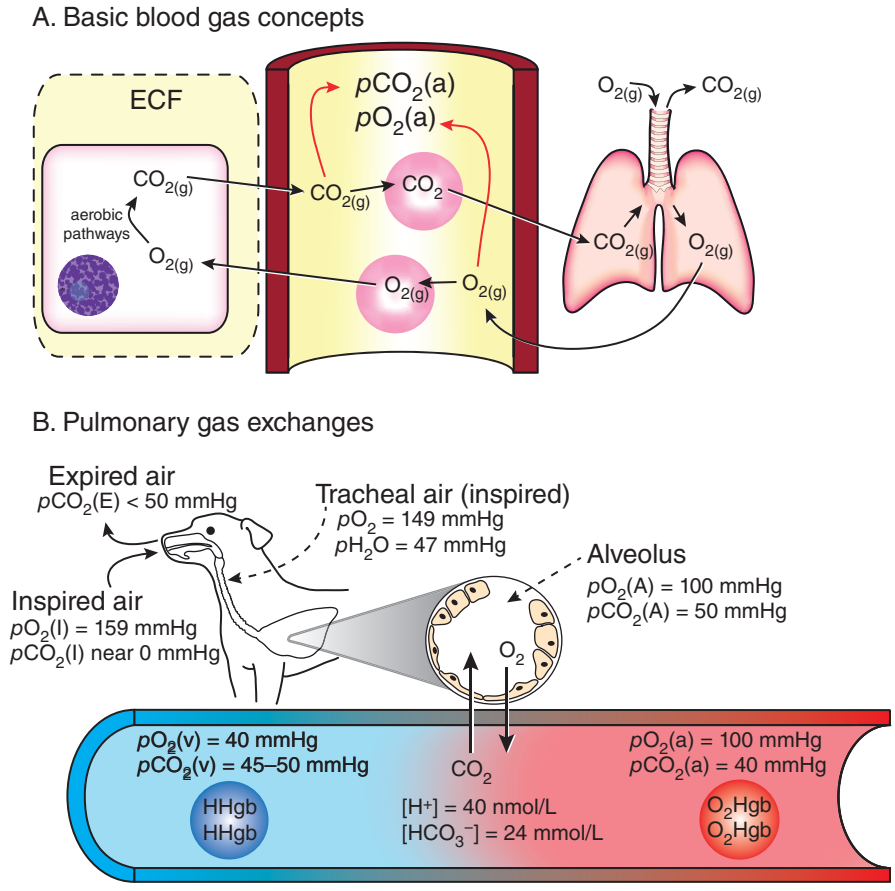


Figure 10.2. Blood gas and pulmonary gas exchanges

A. Basic blood gas concepts: Inhaled O_2 enters pulmonary blood and is transported to tissues, mostly by binding to Hgb in RBCs, but a small amount dissolves in arterial plasma; plasma $O_{2(g)}$ creates the pO_2(a). Cellular aerobic respiration uses O_2 and produces CO_2, which is transported to the lungs, either as dissolved plasma $CO_{2(g)}$ or within RBCs as HCO_3^- (process not shown; see Figure 10.4). The ability of the lungs to remove CO_2 from blood is reflected by blood pCO_2(a).

B. Pulmonary gas exchanges: Schematic drawing of the exchange of O_2 and CO_2 at the alveolus-capillary junction. Pressure values are included in the figure to illustrate the magnitude of changes that occur. Actual pressures vary because of several factors (e.g., total atmospheric pressure is 760 mmHg at sea level but near 735 mmHg at an elevation of 1000 ft).

- Atmosphere to alveolus: pO_2(I) – the pO_2 of inspired air – is 159 mmHg. With the contribution of pH_2O (47 mmHg) in the warm trachea, the pO_2 drops to 149 mmHg: FO_2(I) × (atmospheric pressure – H_2O vapor pressure) = 0.2095 (760 – 47) = 149. In the alveolus, the pO_2(A) is lower, near 100 mmHg, because of the interchange of O_2 with the blood and because of the increased pCO_2(A) (50 mmHg) from the blood.
- Alveolus to blood: In health, O_2 quickly diffuses from the alveolus to the capillary venous blood (pO_2 near 40 mmHg) to give a pO_2(a) of 100 mmHg, which represents the pressure exerted by the dissolved O_2 in plasma. The O_2 also diffuses into RBCs and binds to Hgb (O_2Hgb) to saturate the oxygen-binding sites of ferrous heme (sO_2 = 100 %). With a [Hgb] WRI (e.g., 15 g/dL), 1 L of arterial blood contains about 200 mL O_2 bound to Hgb (i.e., 1.31 mL O_2 per gram of Hgb) and 3 mL of dissolved O_2 that creates the pO_2(a).

2. It may be helpful to consider the bicarbonate system going through H_2CO_3 (Eq. 10.2a), but the actual reaction catalyzed by carbonic anhydrase involves the dissociation of H_2O, release of H^+, and reaction of hydroxide ion (OH^-) with CO_2 to form HCO_3^- without a H_2CO_3 intermediate (Eq. 10.2b). The reaction is reversible.

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow H_2O + CO_{2(g)} \quad \textbf{(10.2a)}$$

$$H^+ + HCO_3^- \xleftrightarrow{\textit{carbonic anhydrase}} H_2O + CO_{2(g)} \quad \textbf{(10.2b)}$$

B. Hyperventilation increases expiration of CO_2 and tends to cause alkalemia as H^+ is removed from plasma. Hypoventilation decreases expiration of CO_2 and tends to cause acidemia as less H^+ is removed via the respiratory path. Two types of chemoreceptors control respiration:
   1. Central chemoreceptors (in the brain stem) are stimulated by increased $[H^+]$ in surrounding fluid.
   2. Peripheral chemoreceptors (carotid and aortic bodies) are stimulated primarily by hypoxemia but also by acidemia (increased $[H^+]$).

C. Oxygenation of blood and pulmonary ventilation
   1. The basic physiologic aspects of the interchange of O_2 and CO_2 are described in Figure 10.2.
   2. For there to be adequate oxygenation of blood, inspired air must have adequate O_2, inspired air must be delivered to alveoli, inspired O_2 must diffuse from alveoli to capillary blood quickly, and the alveoli must be perfused with blood.
   3. For there to be adequate CO_2 loss from pulmonary blood, alveoli must be perfused with blood, CO_2 must diffuse from blood to alveoli quickly, and CO_2 must be expired. Because the solubility of CO_2 in H_2O is greater than that of O_2, the diffusion coefficient of CO_2 is greater, and it can diffuse from blood to alveoli much faster than O_2 can diffuse from alveoli to blood; the diffusion coefficient for CO_2 is 20 times the diffusion coefficient for O_2.

D. Transport of O_2 to tissues
   1. Once O_2 has diffused into pulmonary blood, most of it enters RBCs and binds to Hgb. The processes involved in the oxygenation and deoxygenation of Hgb are illustrated in Figure 10.3.
   2. For there to be adequate oxygenation of tissues, the lungs must oxygenate the blood, blood must have sufficient amounts of functional Hgb, and blood must perfuse the tissues.

Figure 10.2. *continued*

- Blood to alveolus: Reversal of the carbonic anhydrase reaction produces CO_2 (not shown) which quickly diffuses from blood to alveolus and thus lowers the pCO_2(v) to pCO_2(a) near 40 mmHg. The $[H^+]$ decreases slightly because it combines with HCO_3^- to form CO_2 and H_2O. In the healthy lung, CO_2 diffuses from blood to alveoli at 20 times the rate that O_2 diffuses from alveoli to blood.
- Alveolus to atmosphere: When breathing air, the pCO_2(E) is less than the pCO_2(A) because the pCO_2(E) represents a mixture of alveolar gases and the gases in the airways. During anesthesia, measuring the expired pCO_2 (capnography) provides information to assess CO_2 production, pulmonary gas exchange, and elimination of CO_2 by the anesthetic equipment.

pCO_2(E), partial pressure of expired carbon dioxide; pH_2O, partial pressure of water vapor.

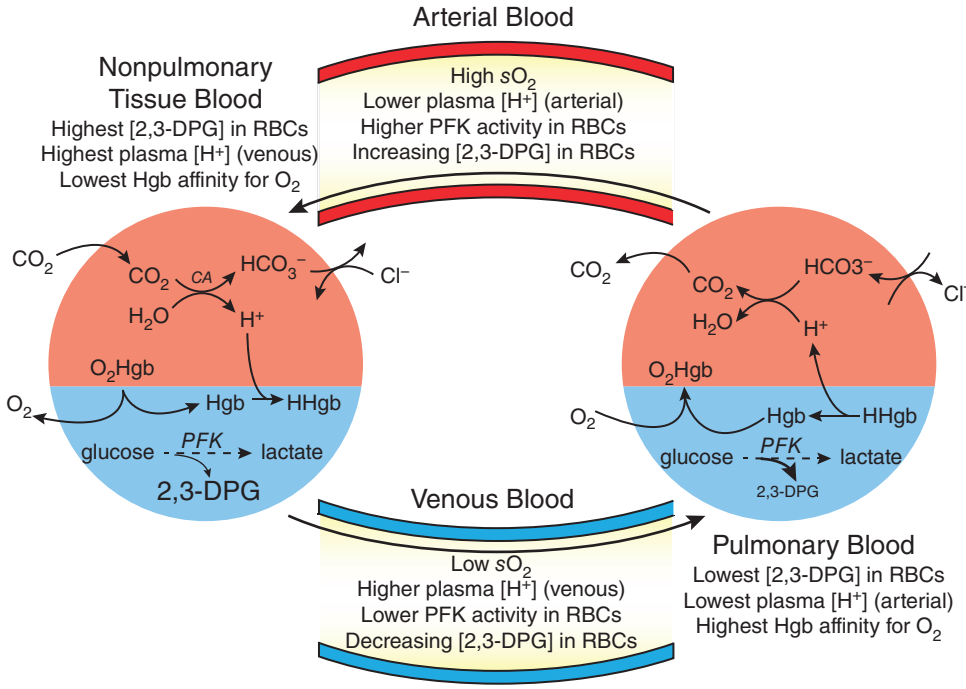


Figure 10.3. Oxygenation and deoxygenation of Hgb. Circles represent RBCs; red portions contain oxyhemoglobin and blue portions contain deoxyhemoglobin.

RBCs in nonpulmonary tissue blood (*left side of figure*)

- Because RBCs have their greatest [2,3-DPG] and greatest [H^+] while moving through capillaries, their Hgb molecules have the lowest affinity for O_2 and thus release O_2 to plasma. The O_2 is then able to diffuse into tissue and participate in metabolic pathways.
- CO_2 (from metabolic pathways) diffuses into plasma and then into RBCs. Via the carbonic anhydrase (*CA*) reaction, CO_2 and H_2O are converted to HCO_3^- and H^+. The HCO_3^- moves to plasma in exchange for Cl^-. Most of the H^+ is buffered by the deoxygenated Hgb (HHgb). The CO_2 produced in tissues is carried in blood in two forms: (i) as dissolved CO_2 with a pCO_2(v) near 45–50 mmHg, and (ii) as HCO_3^- in RBCs and plasma after reacting with H_2O in the presence of carbonic anhydrase.

RBCs in venous blood (*bottom of figure*)

- RBCs leaving tissues have high [2,3-DPG], but the more acidic venous blood (increased [H^+]) inhibits phosphofructokinase (PFK) in the glycolytic pathway. Thus, the rate of 2,3-DPG formation decreases so that RBCs have their lowest [2,3-DPG] when they enter pulmonary blood.

RBCs in pulmonary blood (*right side of figure*)

- O_2 diffuses from alveoli to pulmonary plasma and into RBCs. Because RBCs have their least [2,3-DPG] and least [H^+] in the pulmonary vessels, their Hgb molecules have the greatest affinity for O_2 and thus become saturated with O_2 (sO_2 = 100 %) to form O_2Hgb.
- HCO_3^- moves into RBCs (in exchange for Cl^-) and combines with H^+ to form CO_2 and H_2O; most of the H^+ is buffered by the HCO_3^-. The CO_2 diffuses into plasma and then to alveoli from which it is exhaled.

RBCs in arterial blood (*top of figure*)

- RBCs leaving lungs have low [2,3-DPG] and thus Hgb has high affinity for O_2. The more alkaline arterial blood (lower [H^+]) stimulates PFK in the glycolytic pathway, and thus the rate of 2,3-DPG formation increases so that RBCs have their highest [2,3-DPG] when they enter capillaries in tissues.

IV. Renal functions related to acid-base balance
  A. The kidneys have key roles in maintaining acid-base status (see Chapters 8 and 9).
  B. Renal functions that pertain to acid-base status in health are (i) to excrete H^+, either directly (Figure 9.4) or by incorporation into ammonium (Figure 9.7), dihydrogen phosphate ($H_2PO_4^-$), or bisulfate (HSO_4^-), and (ii) to conserve HCO_3^- (Figure 9.8).
  C. Hormonal and other factors that alter Na^+, Cl^-, and K^+ excretion typically influence renal excretion of H^+ and HCO_3^- (Figures 9.2 and 9.6).

ANALYTICAL CONCEPTS

I. Blood gas instruments measure the $[H^+]$, $pO_2(a)$, and $pCO_2(a)$ in arterial blood samples. However, the analytes are in the blood's plasma. They also measure $[H^+]$, $pO_2(v)$, and $pCO_2(v)$ in venous samples (see Section V.B for differences between arterial and venous samples).
  A. Measured analytes and methods
    1. Descriptions of measured analytes
      a. $[H^+]$ is the free $[H^+]$ in plasma. It reflects the net effect of bodily processes on plasma $[H^+]$. It is reported as pH, the unitless –log of the $[H^+]$; a pH of $7.4 = 10^{-7.4}$ mol/L = 40 nmol/L of H^+.
      b. $pO_2(a)$ (mmHg) is the partial pressure of O_2 in arterial plasma. It reflects the amount of dissolved $O_{2(g)}$; it does not measure O_2 associated with Hgb. It is useful in assessing pulmonary gas exchange.
      c. $pO_2(v)$ (mmHg) is the partial pressure of O_2 in venous plasma. Its value depends on oxygenation of pulmonary blood and the use of O_2 in tissues.
      d. $pCO_2(a)$ (mmHg) is the partial pressure of CO_2 in arterial plasma. It reflects the amount of dissolved $CO_{2(g)}$ in plasma and is useful to assess alveolar ventilation.
      e. $pCO_2(v)$ (mmHg) is the partial pressure of CO_2 in venous plasma. It may be useful to assess alveolar ventilation if there is adequate blood perfusion of lungs and tissues.
      f. Unit conversion for pO_2 and pCO_2: mmHg × 0.133 = kPa (kilopascal, International System unit)
    2. Blood gas analyzers used for veterinary patients were designed to be used for human samples. Therefore, measurements are made at 37 °C rather than at the temperatures of typical veterinary patients, and values are not accurate reflections of the in vivo values (see Section I.C, Temperature correction factors).
    3. Selective electrodes measure pH and pCO_2 by potentiometry, detecting voltage differences generated by H^+ gradients. For the pH electrode, the H^+ gradient forms as H^+ in the test sample interacts with the electrode's membrane and causes H^+ on the inner membrane to shift to the inner fluid. In the pCO_2 system, CO_2 passing through the electrode membrane reacts in a bicarbonate buffer to change the pH; the change in pH is detected by a pH (H^+-selective) electrode.
    4. pO_2 is measured by amperometry, which detects the flow of electrons generated as O_2 is reduced to H_2O after diffusing across the membrane of an O_2-selective electrode.
    5. The membranes also protect the electrodes from proteins and other substances in the plasma. Maintenance of blood gas instruments involves maintenance (cleaning and replacement) of the membranes.
    6. Some blood gas instruments measure sO_2 by spectrophotometry based on the differences in the absorption of different wavelengths of light by O_2Hgb and HHgb. In most analyzers, sO_2 is calculated.

7. Point-of-care testing: Small hand-held or portable chemical analyzers that use a disposable cartridge containing ion-selective electrodes are designed to operate at 37 °C and can do the following: (i) measure blood gas values, i.e., pH, pCO_2, and pO_2, (ii) measure common plasma analyte concentrations (Na^+, K^+, Cl^-, urea, creatinine, glucose, fCa^{2+}, and Hgb), and (iii) calculate several concentrations or values (HCO_3^-, tCO_2, AG, sO_2, $BE_{(ecf)}$, BE(B), and hematocrit). Comparisons with laboratory blood gas analyzers have shown inaccuracies for certain measurands in certain species; published relevant studies can be evaluated when considering use of a particular analyzer. Because of analyzer biases, it is best to do serial testing on a single analyzer.

B. Calculated acid-base and blood gas values: The following values can be calculated from the measured pH, pCO_2, and pO_2 by using other known factors (e.g., body temperature or [Hgb]). Clinically, the three calculated values discussed below after "Buffer base" are used to assess pulmonary function, especially during anesthesia.
   1. $[HCO_3^-]_{actual}$ (in mmol/L) is calculated via the Henderson–Hasselbalch equation (Eq. 10.1a) by using the measured pH and measured pCO_2.
   2. $[tCO_2]$ (in mmol/L) is calculated from the calculated $[HCO_3^-]_{actual}$ and measured pCO_2 (Eq. 10.3). The pCO_2 is multiplied by 0.0307 (a solubility coefficient) to calculate the amount of dissolved CO_2 in the blood sample. The $[tCO_2]$ generated by routine serum chemistry analysis is based on different methods.

$$[tCO_2] = [HCO_3^-] + [H_2CO_3] = [HCO_3^-] + (pCO_2 \times 0.0307) \quad (\mathbf{10.3})$$

   3. $[HCO_3^-]_{standard}$ (in mmol/L) is also called standard bicarbonate concentration (SBC).
      a. $[HCO_3^-]_{standard}$ is the $[HCO_3^-]$ in plasma when fully oxygenated blood from a "normal" animal is equilibrated at 37 °C to a pCO_2 of 40 mmHg. The calculated $[HCO_3^-]_{standard}$ is an estimate of the $[HCO_3^-]$ in the sample if the pCO_2 were 40 mmHg. However, there are species and individual differences in pCO_2 that, if accounted for by the calculation, would yield different and more applicable $[HCO_3^-]_{standard}$ estimates.
      b. In disease, the $[HCO_3^-]_{standard}$ is theoretically a better estimate of buffer base in a blood sample because it is independent of changes in pCO_2, whereas a $[HCO_3^-]_{actual}$ depends on pCO_2 values (e.g., when pCO_2 increases, $[HCO_3^-]$ slightly increases in plasma).[3]
   4. $BE_{(ecf)}$ or BE(P) (in mmol/L)
      a. $BE_{(ecf)}$ or BE(P) is the amount of strong acid (mmol/L) that is needed to titrate extracellular fluid (for $BE_{(ecf)}$) or plasma (for BE(P)) to a pH of 7.4 if the pCO_2 is 40 mmHg at 37 °C.
      b. The calculation uses the measured pH, calculated $[HCO_3^-]$, an assumed "normal" $[HCO_3^-]$ (e.g., 24–25 mmol/L), and an assumed "normal" plasma protein concentration. These assumptions are not valid for all species, all individuals, or venous samples.
      c. The $BE_{(ecf)}$ and HCO_3^- values provide the same diagnostic information in most metabolic disorders and can be considered interchangeable.[4]
      d. If the assumptions are correct, and often they are not:
         (1) A positively increased $BE_{(ecf)}$ indicates an excess of plasma buffer (e.g., HCO_3^-, proteins) and thus a metabolic alkalosis (base-excess definition).
         (2) A decreased (more negative) $BE_{(ecf)}$ indicates a deficit of plasma buffer (e.g., HCO_3^-, proteins) and thus a metabolic acidosis (base-excess definition).

(3) If blood pH changes because of a change in pCO_2 but the [HCO_3^-] is WRI, then the $BE_{(ecf)}$ remains WRI. This would occur in respiratory acidoses or alkaloses when there has not been sufficient time for metabolic compensation.

5. BE(B) (in mmol/L)
   a. The BE(B) is the amount of strong acid (mmol/L) that is needed to titrate whole blood to a pH of 7.4 if the pCO_2 is 40 mmHg at 37 °C.
   b. The calculation involves the same values as $BE_{(ecf)}$ plus the contribution of Hgb to the buffer base. RBC 2,3-DPG contributes to the buffer base but is not specifically included in the calculation. The Hgb value may be inaccurate because of mean cell Hgb concentration (MCHC) assumptions when calculated from measured hematocrit values, or because of inaccuracies of the blood gas analyzer's hematocrit calculation (see Chapter 3).
   c. Increased (more positive) and decreased (more negative) BE(B) values provide essentially the same information about the patient as does the $BE_{(ecf)}$. The BE(B) values should approach the $BE_{(ecf)}$ as an animal becomes more anemic.
6. Buffer base (or total buffer base) (in mmol/L)
   a. Buffer base is the sum of the concentrations of "buffer ions" in blood, including HCO_3^-, Hgb, PO_4, and plasma proteins. Buffer base in plasma does not include Hgb.
   b. Buffer base is related to base excess as follows: buffer $base_{actual}$ = buffer $base_{normal}$ + base excess. Buffer $base_{normal}$ is the expected sum of the "buffer ions" in a healthy human. Base excess (if positive) is the amount of excess "buffer ions" compared to normal.
   c. SID is similar to buffer base as introduced in 1948 by Singer and Hastings.[3]
7. sO_2 (in %)
   a. sO_2 is the estimated amount of O_2Hgb in blood expressed as a percentage of the total amount of Hgb able to bind O_2 (i.e., not intended to include metHgb or COHgb, which are not able to bind O_2 and do not affect sO_2 despite decreasing blood oxygen content). sO_2 should not be confused with FO_2Hgb (fraction of O_2Hgb), which is a fractional expression of O_2Hgb in blood expressed as a percentage of the total amount of Hgb (total includes Hgb molecules that do not bind O_2) (Eq. 10.7d). See Pulse oximetry, Section II, and CO-oximeter, Section III, for methods of measuring the percentage.
   b. The sO_2 depends primarily on the pO_2 as described by the sigmoid O_2-Hgb dissociation curve (Figure 10.4). At a pO_2(a) of 100 mmHg, the sO_2 is expected to be about 97 %. Acidemia, increased RBC [2,3-DPG], hyperthermia, and hypercarbia shift the dissociation curve to the right, meaning that sO_2 decreases for any given pO_2.
   c. Traditional blood gas instruments calculate the sO_2 from measured pH, pCO_2, and pO_2 values by using formulas that attempt to correct for temperature variations and to describe the O_2 dissociation curve.[8] Assuming that the O_2 affinity for Hgb and [2,3-DPG] are those found in health, the following relationships apply.[9]
      (1) If the pH is constant, but the pO_2 decreases, then sO_2 decreases.
      (2) If the pO_2 is constant, but the pH decreases (acidemia), then sO_2 decreases. If pO_2 is constant, but pH increases (alkalemia), then sO_2 increases.
   d. However, assumptions for sO_2 calculations may not be met in hospitalized patients with altered [2,3-DPG] or other abnormalities, so calculated sO_2 and further derived

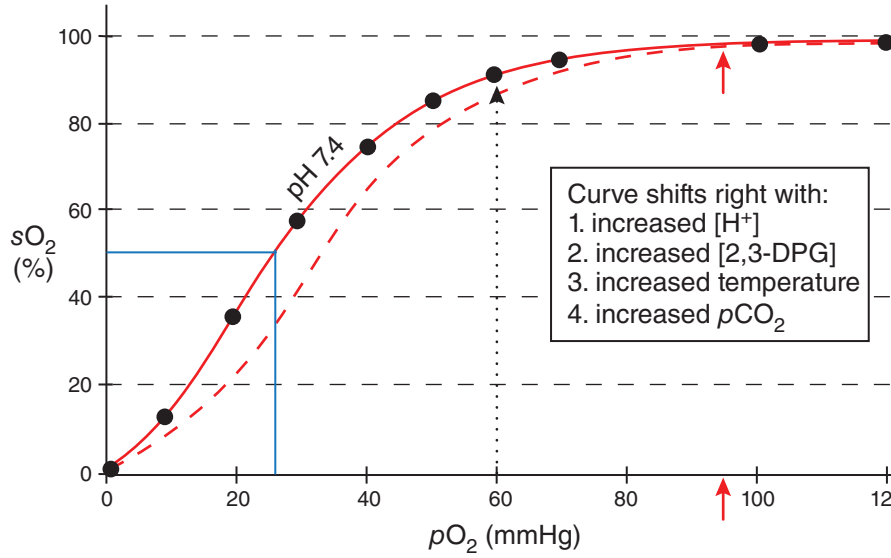


Figure 10.4. Oxygen-hemoglobin dissociation curve. With a typical pO_2(a) of 95 mmHg and at a pH of 7.4, the sO_2 is near 96 %) (*red arrows*). Because of the high affinity of Hgb for O_2, the sO_2 remains > 90 % as long as the pO_2 remains > 60 mmHg (*dotted arrow*). When pO_2 values are > 100 mmHg, the sO_2 is near 100 %. Increased [H^+], increased RBC [2,3-DPG], hyperthermia, and hypercarbia shift the curve to the right (*dashed red curve*). If pO_2 stays constant, shifting the curve to the right results in a lower sO_2. The displayed dissociation curve was constructed from human data. Dissociation curves for dogs[5] and other species are slightly different because of differences in Hgb molecules, [2,3-DPG], and other factors. Accordingly, the average p_{50} values – the pO_2(a) when hemoglobin is 50 % saturated with oxygen at pH 7.4, 37 °C, and 40 mmHg pCO_2(a) – differ: horses ≅ 25 mmHg, cattle ≅ 26 mmHg, people ≅ 27 mmHg (*blue lines*), dogs ≅ 30 mmHg, and cats ≅ 34 mmHg.[6,7]

values may be unreliable. sO_2 may also be determined by multiwavelength oximetry (see Section II.C below).

8. pO_2(A) (in mmHg): alveolar O_2 pressure (sometimes abbreviated as A)
   a. pO_2(A) is the partial pressure of O_2 in the alveolar air (gas). Two formulas incorporating FO_2(I) and pCO_2(a) can be used to calculate the value (Eq. 10.4a).[8]
      (1) The calculated pO_2(I) is dependent on the local atmospheric (barometric) pressure and FO_2(I) (Eq. 10.4b). Current blood gas analyzers can measure the local atmospheric pressure during the analysis of a blood gas sample and include it in the calculation.
      (2) FO_2(I) is the fractional percentage of inspired O_2 in a gas. The FO_2(I) of air is 0.2095 (or about 21 %); the pO_2(I) of air at sea level is about 159 mmHg (20.95 % of 760 mmHg), but it is about 134 mmHg (20.95 % of 640 mmHg) at Denver's altitude. During gas anesthesia, the FO_2(I) could be 1.0 (100 % O_2).
      (3) For a given FO_2(I) and a constant metabolic respiratory quotient, the calculated pO_2(A) is lower at higher altitudes, as is the pCO_2(a).
      (4) The pO_2(A) calculation assumes that pCO_2(a) = pCO_2(A), an assumption that may not be true if there is a mismatch in ventilation–perfusion.

$$\text{Simplified: } pO_2(A) = pO_2(I) - \frac{pCO_2(a)}{R} \qquad (\mathbf{10.4a})$$

$$\text{Complete: } pO_2(A) = pO_2(I) - \frac{pCO_2(a)}{R}\ (1 - FO_2(I)(1 - R))$$

where $pO_2(A)$ is partial pressure of alveolar oxygen, $pO_2(I)$ is the partial pressure of inspired oxygen, $pCO_2(a)$ is the partial pressure of CO_2 in arterial blood, R is the average metabolic respiratory quotient, and $FO_2(I)$ is the fractional concentration of oxygen in inspired air (gas).

$$\text{Partial pressure of inspired oxygen} = pO_2(I) = (p(\text{Atm}) - p(H_2O)) \times FO_2(I) \qquad (\mathbf{10.4b})$$

where $p(\text{Atm})$ is atmospheric (barometric) pressure, $p(H_2O)$ is H_2O vapor pressure in the airway, and $FO_2(I)$ is the fractional concentration of oxygen in inspired air (gas).

b. The respiratory quotient (R) (in Eq. 10.4a) is the ratio of the volume of CO_2 produced to the volume of O_2 consumed by a body. In other words, R is the ratio of the amount of CO_2 diffusing from blood into alveoli to the amount of O_2 diffusing in the opposite direction. The respiratory quotient varies with the type of fuels the body is using (e.g., fats versus carbohydrates). Generally, the average ratio is 0.8, meaning that 0.8 L of CO_2 is formed when 1 L of O_2 is consumed by metabolic pathways. However, the value for R can vary considerably among species and within and among individuals of the same species.

c. The concentration of alveolar O_2 for a given concentration of O_2 in inspired air is determined by a balance between alveolar ventilation and the amount of O_2 diffusing into capillary blood.

9. $DO_2(A/a)$, often referred to as the *A–a gradient*, is the alveolar–arterial O_2 tension gradient (in mmHg) (Eq. 10.5a).

a. The value is used as an index of the exchange of O_2 in lungs, i.e., the delivery (D) of alveolar O_2 to arterial blood. Increasing values are found with right-to-left shunts of blood around the lungs or other causes of ventilation–perfusion mismatches, and less commonly with impaired diffusion of oxygen from alveoli to capillaries.

b. It should be noted that the calculated $DO_2(A/a)$ is derived from a calculated $pO_2(A)$, which is derived from a calculated $pO_2(I)$ (see Eq. 10.4b) that may factor in actual measured local barometric pressure, in which case clinical adjustment of reported values for local atmospheric pressure may be inappropriate.

$$\text{Alveolar–arterial oxygen tension gradient} = DO_2(A/a) = pO_2(A) - pO_2(a) \qquad (\mathbf{10.5a})$$

(or A – a when $pO_2(A)$ is abbreviated "A" and $pO_2(a)$ is abbreviated "a")

$$\text{Arterial–alveolar oxygen tension ratio: } a/A = \frac{pO_2(a)}{pO_2(A)} \qquad (\mathbf{10.5b})$$

$$\text{Inspired oxygen fraction ratio: } pO_2(a)/FI = \frac{pO_2(a)}{FO_2(I)} \qquad (\mathbf{10.5c})$$

10. a/A (unitless) is the arterial–alveolar O_2 tension ratio (Eq. 10.5b). The value is used as an index of the exchange of O_2 in lungs; unlike the A–a gradient, it remains relatively stable with changes in $FO_2(I)$.
11. $pO_2(a)/FI$ (unitless) is the inspired O_2 fraction ratio (Eq. 10.5c). The value is used as an index of the delivery of O_2 to alveoli and the exchange of O_2 in lungs.
12. O_2ct (mL/dL) is the O_2 content. The O_2ct represents the total amount (volume) of O_2 in a given blood volume (100 mL), including that dissolved in plasma and that bound to Hgb.
13. p_{50}, also referred to as $pO_2(a)(0.5)$ (in mmHg): The p_{50} is defined as the $pO_2(a)$ of a sample when Hgb is 50 % saturated with O_2 at pH 7.4 and 37 °C, with a $pCO_2(a)$ of 40 mmHg (Figure 10.4).

C. Temperature correction factors for blood pH and gas values with variations in body temperature
1. Partial pressures of dissolved gases vary with the temperature of the solvent. For example, if O_2 and CO_2 content remain constant in a sample but temperature increases, the pO_2 and pCO_2 values increase (hot gas expands and thus exerts more pressure).
2. Blood gas instruments measure pH, pO_2, and pCO_2 in a 37 °C chamber. If the patient's temperature is 40 °C, then the in vivo values are different from those determined by the instrument. Correction factors for increasing temperatures are in Table 10.2, which shows, for example, that if the pCO_2 were 40 mmHg at 37 °C, then the pCO_2 would be 44 mmHg (40 + 10 % of 40 mmHg) at 39 °C and 48 mmHg (40 + 20 % of 40 mmHg) at 41 °C.
3. There are differences of opinion and unanswered questions regarding the need for temperature correction of blood gas values. Corrections only adjust for the physical changes in the sample but do not account for metabolic or cardiovascular changes that may occur with temperature changes.
   a. For example, lowering blood's temperature (as in hypothermia) lowers the $pO_2(a)$, but the O_2 content of blood does not change. In the hypothermic state, is the lower $pO_2(a)$ value desired for the lower metabolic rate or should the $pO_2(a)$ be increased by increasing the $FO_2(I)$?
   b. If temperature-corrected values are used to monitor a patient, and if the patient's body temperature changes, will changes in pH, $pO_2(a)$, or $pCO_2(a)$ be caused by changes in body temperature or other factors?
4. If a laboratory reports temperature-corrected values, it should also report the values measured at 37 °C. If all in vitro values are reported at 37 °C regardless of the body temperature, then changes in pO_2 and pCO_2 values reflect changes in O_2 and CO_2 content of plasma. Most likely, RIs for the data are generated with measurements at 37 °C and not corrected for temperature variations among species.

Table 10.2. Correction of blood gas and pH values for variations in body temperature

°C	°F	pH	pCO_2	pO_2
41	105.8	– 0.06	+ 20 %	+ 33 %
40	104.0	– 0.04	+ 15 %	+ 24 %
39	102.2	– 0.03	+ 10 %	+ 15 %
38	100.4	– 0.01	+ 5 %	+ 7 %
37	98.6	None	None	None

Note: Correction factors calculated from established formulas[10]

II. Pulse oximetry

A. An *oximeter* is a device that spectrophotometrically measures arterial sO_2, and, when determined via pulse oximetry through tissue containing pulsating vessels, it is denoted as spO_2 (p to indicate *pulse*) and meant to represent the percentage of *all* Hgb that is O_2Hgb. During the 1980s, the pulse oximeter became an accepted instrument in human medicine for monitoring Hgb oxygenation during anesthesia and is common in the monitoring of critical care patients. The original medical pulse oximeters used two wavelengths of transmitted light; more recent versions use multiple wavelengths of transmitted light or use reflectance photometry.

B. Two-wavelength pulse oximeter

1. The pulse oximeter determines the spO_2 by transmission or reflectance spectrophotometric methods.[11,12] Light at one red and one infrared wavelength (e.g., 660 and 940 nm) is passed through tissue that contains arterial blood (e.g., the ear lobe or finger in people, the tongue in horses, and the tongue, lip, ear, toe, tail, prepuce, or vulva in dogs).[13,14] In dogs, the best agreement of spO_2 with sO_2(a) occurred with the lip and tongue placements.[15]
2. At the infrared wavelength (e.g., 940 nm), O_2Hgb absorbs more light than does HHgb (reduced Hgb). At the lower wavelength (e.g., 660 nm), O_2Hgb absorbs much less light than does HHgb. If severe anemia (Hct < 10 %) is present, pulse oximeter values are unreliable because the method relies on a sufficient amount of Hgb to obtain different absorbance readings for O_2Hgb and HHgb.[16]
3. The pulsatile change in red and infrared absorbance occurring as arterial blood enters the tissue in systole is measured in comparison to the nearly constant red and infrared absorbance of venous blood and other tissues. This yields the *red to infrared modulation ratio*, R (Eq. 10.6), which is calibrated to direct measurements of sO_2(a) in healthy individuals having saturation from about 70 % to 100 %.[11,17] R is inversely related to spO_2, being approximately 0.4 with 100 % of the Hgb saturated, 1.0 with about 85 % saturation, and about 3.4 with 0 % saturation.

$$\text{Red to infrared modulation ratio} = R = \frac{A_{660,\text{pulse}}/A_{660,\text{nonpulse}}}{A_{940,\text{pulse}}/A_{940,\text{nonpulse}}} \qquad (\mathbf{10.6})$$

where A660 and A940 are absorbances at 660 nm (red) and 940 nm (infrared), respectively, nonpulse absorbance is the constant absorbance of tissue and venous blood, and pulse absorbance is the change in absorbance amplitude caused by the influx of arterial blood during systole.

4. Calibrations using human beings may not be appropriate for use with domestic mammals.
   a. In horses, the spO_2 was less than the sO_2(a) by an average of 4.4 % when sO_2(a) was $\geq$ 90 %, and greater than the sO_2(a) by an average of 4.1 % when sO_2(a) was $\leq$ 90 %.[18]
   b. In dogs with $sO_2(a) \geq 70$ %, the average difference between spO_2 and sO_2(a) was about 3–4 percentage points (e.g., spO_2 of 87 % and sO_2(a) of 90 %), depending on application site (tongue or tail) and type of probe (ear or finger). The reason for the difference is not known.[13] As with the equine data, spO_2 was lower than the sO_2(a) at high saturations, and greater than the sO_2(a) when it was at low saturations.

5. The interference caused by other light-absorbing substances in the tissues (including melanin) is reduced (not eliminated) by measuring the light while arterial blood pulses through the tissue (i.e., only pulsatile changes in absorption are measured, which could include those from an exogenous dye like methylene blue, but icterus does not contribute). Accurate measurements require an adequate pulse, so hypotension and poor perfusion can falsely reduce $sp\mathrm{O}_2$. Measurement interference also can occur with shivering, other movements, and ambient light (reflectance method).
6. The association between $sp\mathrm{O}_2$ and the red to infrared modulation ratio is based on the presence of very low percentages of COHgb and metHgb in health. Increases in either type of Hgb in diseased animals cause inaccurate results because of their effects on red and infrared light absorption.
   a. Carboxyhemoglobinemia decreases the pulsatile red to infrared modulation ratio, thus falsely increasing the reported $sp\mathrm{O}_2$ by a percentage increment that is similar to the COHgb percentage increase. The $sp\mathrm{O}_2$ may remain above 90 % even when there are lethal amounts of COHgb.
   b. Methemoglobinemia increases the pulsatile red to infrared modulation ratio until it plateaus at 1.0 (with 30 % metHgb); the ratio plateaus because metHgb has the same absorption at red and infrared wavelengths. For a patient starting with a $s\mathrm{O}_2(a) >$ 85 %, the effect of increasing metHgb is to decrease the $sp\mathrm{O}_2$; $sp\mathrm{O}_2$ will then underestimate $s\mathrm{O}_2(a)$. With more than 30 % metHgb, the $sp\mathrm{O}_2$ remains at about 85 %, thus overestimating $sp\mathrm{O}_2$ and either overestimating or underestimating $s\mathrm{O}_2$, depending on $p\mathrm{O}_2(a)$.
   c. Both carboxyhemoglobinemia and methemoglobinemia decrease oxygen-carrying capacity of blood and cause inaccuracies in $sp\mathrm{O}_2$, but they do not decrease $s\mathrm{O}_2$ as measured by traditional blood gas analyzers or nonpulsatile multiwavelength oximeters (see Hypoxemia and Hypoxia, Section II.D).

C. Multiwavelength pulse oximeters (also called pulse CO-oximeters and pulse carbon monoxide oximeters)
   1. Like the CO-oximeters described in the next section, these pulse oximeters use seven or more wavelengths of light to identify the presence of COHgb and metHgb in blood, thus yielding reasonably accurate $sp\mathrm{O}_2$ and $F\mathrm{O}_2\mathrm{Hgb}$ when these dyshemoglobinemias are present in people.[11] Few studies have assessed pulse CO-oximetry in domestic mammals, and most have related to monitoring anesthetized dogs and horses.
   2. A pulse CO-oximeter has detected methemoglobinemia in a dog with a low $sp\mathrm{O}_2$ based on initial conventional pulse oximetry.[19] When dyshemoglobinemia is suggested by pulse CO-oximetry, laboratory confirmation is recommended prior to treatment.

III. CO-oximeters (also called multiwavelength and carbon monoxide oximeters)
   A. As with a multiwavelength pulse oximeter, a CO-oximeter determines the different types of Hgb by measuring the absorbance of several wavelengths of light, but by using blood samples removed from the patient. Each type of Hgb has a unique absorbance spectrum, and the concentration of each type is determined. The CO-oximeter can be used to identify methemoglobinemia and CO poisoning.
   B. CO-oximetry is also called Co-oximetry, co-oximetry, and cooximetry.
   C. Traditionally, RBCs in the sample were lysed prior to analysis, but some instruments use whole blood with methods to minimize or mathematically correct for RBC-induced light scattering.

D. Calculated values (Eq. 10.7)

$$[tHgb] = [O_2Hgb] + [HHgb] + [COHgb] + [MetHgb] \quad (\mathbf{10.7a})$$

$$O_2Hgb\% = \frac{[O_2Hgb]}{[tHgb]} \times 100 \qquad HHgb\% = \frac{[HHgb]}{[tHgb]} \times 100$$
$$COHgb\% = \frac{[COHgb]}{[tHgb]} \times 100 \qquad MetHgb\% = \frac{[MetHgb]}{[tHgb]} \times 100 \quad (\mathbf{10.7b})$$

$$sO_2 = \frac{[O_2Hgb]}{[O_2Hgb] + [HHgb]} \times 100 \quad (\mathbf{10.7c})$$

$$FO_2Hgb = \frac{[O_2Hgb]}{[tHgb]} \quad (\mathbf{10.7d})$$

$$O_2ct = \frac{1.31 \text{ mL } O_2}{\text{g Hgb}} \times [tHgb] \times \frac{sO_2}{100} \quad (\mathbf{10.7e})$$

1. Total Hgb (tHgb) concentration represents the sum of the Hgb types as determined by absorbance photometry (Eq. 10.7a).
2. The percentages of each type of Hgb are calculated from the concentrations determined by absorbance photometry (Eq. 10.7b).
3. CO-oximeter sO_2 values are accurate in the presence of methemoglobinemia and carboxyhemoglobinemia (the saturation of the Hgb that can carry oxygen is not decreased), but sO_2 is not equivalent to FO_2Hgb, which would be decreased (Eq. 10.7c–d).
4. O_2ct is calculated from the oximeter's measured values (Eq. 10.7e).

E. Blood substitutes may interfere with CO-oximeter values. Compared to results obtained from an O_2-selective electrode assay, the O_2 content measured by an Instrumentation Laboratories CO-oximeter differed more than 20 % 1 h after patient infusion with a Hgb-based O_2 carrier. The differences were greater with the larger dosage of the blood substitute.[20] However, The NOVA CO-Oximeter provided accurate values for O_2 saturation with various concentrations of Hgb-based O_2 carriers.[21]

IV. Samples for blood gas and pH analysis

A. As stated in the blood gas measurement guidelines of the Clinical and Laboratory Standards Institute, "Blood gas measurements for the purpose of evaluating the gas exchange function of the lungs (pO_2 and pCO_2) should be performed on arterial blood only."[1] Unfortunately, laboratories may be unable to provide appropriate arterial blood gas RIs given difficulties and concerns related to collecting arterial blood from healthy reference individuals, and published RIs may not be appropriate for your patient population or analyzer.[22,23] If published arterial and venous RIs were determined appropriately and concurrently, and if the venous RIs are similar to appropriate venous RIs for another laboratory lacking arterial RIs, then the reported arterial RIs can provide guidance for data from the other laboratory.

B. Arterial versus venous blood samples

1. Arterial blood samples are rarely collected if an animal is not anesthetized, and thus venous samples are commonly assessed clinically.
   a. Metabolic acid-base disorders are much more common than respiratory disorders in spontaneous clinical disease. For most animals with metabolic disorders, there

is not a concurrent pulmonary disease that would affect gas exchange, so looking for respiratory compensation (or assessing pCO_2) is not needed.

b. As noted in several tables and figures, changes in pCO_2(v) may reflect pCO2(a) but less reliably, especially with circulatory failure or during mechanical ventilation.

2. A comparison of arterial and jugular venous blood samples from clinically healthy cats yielded a mean pO_2(a) that was 65 mmHg greater than the pO_2(v), with a 95 % confidence interval of 45–85 mmHg. The mean pCO_2(a) was 6 mmHg less than the pCO_2(v), with a 95 % confidence interval of 0–12 mmHg.[24] Similar findings have been published for human blood samples collected prospectively in a standardized manner and promptly analyzed in duplicate to reduce effects of assay imprecision.[25]
3. Similar discordant results were obtained when blood gas data from paired femoral arterial and central venous blood samples from 46 dogs were compared (Figure 10.5).[26] Note: Central venous samples are a combination of venous blood from a variety of tissues.
   a. To assess if central venous samples could reliably be used to assess the acid-base status in ill dogs, the authors used linear regression equations to predict arterial values from venous data; they then compared the actual and predicted values and calculated their differences.
   b. Seven of 46 pH comparisons differed by more than 0.034, and two differed by more than 0.068, which exceeds the Clinical Laboratory Improvement Amendments (CLIA) current allowable total error of ± 0.04 for pH of human samples.[27]
   c. Seven of 46 pCO_2 comparisons differed by more than 4 mmHg, one by more than 8 mmHg, which exceeds the CLIA allowable total error of ± 5 mmHg for pCO_2 in human samples.[27]
   d. Four of 46 HCO_3^- comparisons differed by more than 1.6 mmol/L; none differed by more than 3.3 mmol/L.
4. Venous [HCO_3^-] and pCO_2(v) may be used for classifying most metabolic and many respiratory acid-base disorders if collected anaerobically and appropriately processed.
   a. However, even if valid RIs are available for pCO_2(v) (including central venous pCO_2), one should be careful interpreting the patient data; a pCO_2(v) may not accurately reflect pulmonary function because of variable CO_2 production by a patient's tissues; this contributes to the variable differences between pCO_2(a) and pCO_2(v) (Figure 10.5).
   b. In few cases, venous data may help identify circulatory failure when used in conjunction with arterial data.[28] Large arterial–venous pCO_2 differences have been found in human patients with severe circulatory failure, during mechanical ventilation, and when tissues are hypoperfused. The pCO_2(v) may increase because of increased tissue CO_2 production and decreased CO_2 delivery to lungs, but the pCO_2(a) does not reflect those changes because CO_2 was removed from the blood that actually passed through the lungs.[29]

C. Arterial versus capillary blood samples
1. In human medicine, when arterial blood cannot be collected, "arterialized" capillary blood can provide values that approximate blood gas data of an arterial blood sample.[1] The arterialized capillary blood is collected from free-flowing blood (without tourniquet) from skin after the area has been warmed to increase blood flow.
2. Evaluation of capillary samples in dogs
   a. Two sets of blood samples from incisions near the tips of tails and from femoral arteries were collected from 12 healthy dogs; the tail was not warmed prior to collection. The values for pH, pCO_2, and [HCO_3^-] were nearly the same; pO_2 was not measured.[30]

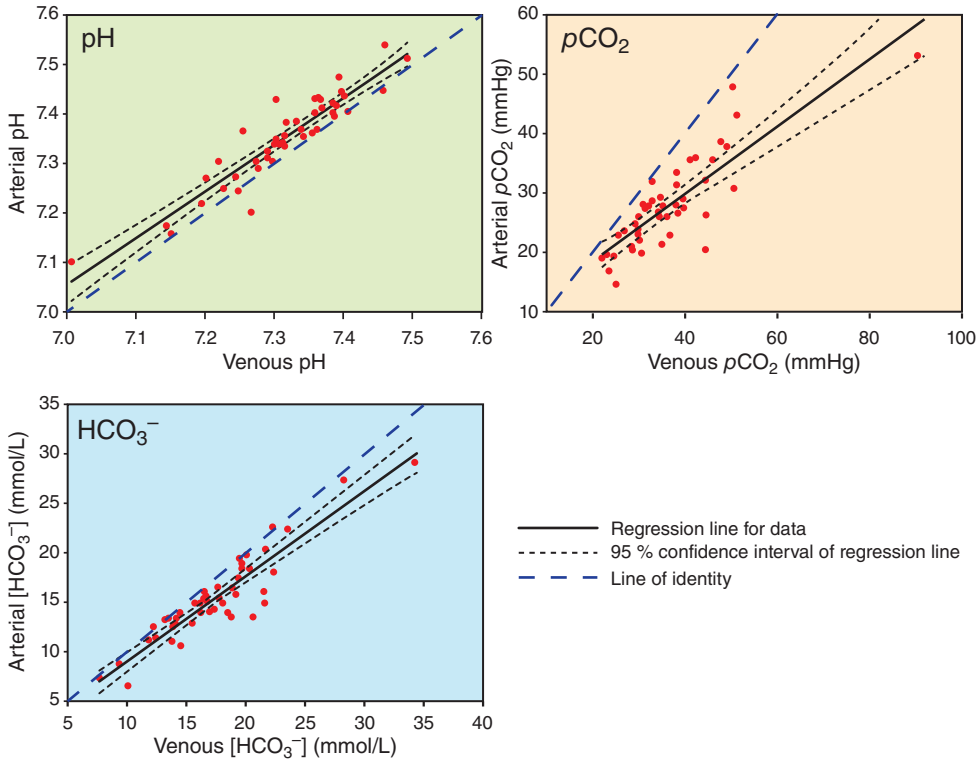


Figure 10.5. Comparison of acid-base data from paired arterial and central-venous blood samples. Samples were collected from 46 nonanesthetized dogs with a variety of disorders excluding cardiopulmonary arrest, and venous samples were central venous samples and therefore a combination of venous blood from a variety of tissues. Each sample was tested once. These graphs were recreated from the published data.[26]

- *Top left*: Venous pH values are more acidic than arterial pH values as shown by constant bias (the 95 % confidence interval of the regression line does not include the *y*-intercept of the line of identity, but the regression line is nearly parallel to the line of identity). A few data points show a pH difference near 0.1. Venous blood is more acidic because H^+ is produced by tissues and is being transported to lungs and eventually to kidneys for removal.
- *Top right*: Regression line shows a large proportional bias with venous pCO_2 values greater than arterial pCO_2 values and greater differences at greater pCO_2(a) values. The amount of scatter in the data illustrates that it would be difficult to predict pCO_2(a) from pCO_2(v).
- *Bottom left*: Regression line shows a small proportional bias with most venous HCO_3^- values greater than arterial values; greatest incremental differences occur at greater HCO_3^- concentrations.

b. Arterial (femoral), venous (femoral), and capillary (ear) blood samples were collected from four anesthetized healthy dogs with induced states of hypoxemia, hypercapnia, and hypovolemia; the ears were not warmed. The authors concluded that capillary (ear) and arterial blood samples had similar blood gas values (pH, pCO_2, pO_2) for hypoxemic and hypercapnic states, but not when then there was impaired blood circulation due to hypovolemia.[31] Similar findings were found in other dogs.[32]

c. Blood samples from aorta and toenails of 10 healthy, anesthetized dogs provided comparable results for pH, pCO_2, and $[HCO_3^-]$.[33]

3. Cats: Forty-six, paired samples of arterial (femoral) and "arterialized" blood from cut claws were collected from seven healthy cats. To "arterialize" the blood, a paw was placed in a warm (42–45 °C) 60 mL syringe barrel for at least 10 min. The pO_2(a) and pO_2(v) did not differ significantly. The pH values of the "arterialized" blood were mildly but significantly greater than those of the arterial blood; the reason for this was not clear, but there were several differences between paired samples other than arterialization which might have affected the comparison (collection site, volume, container, anticoagulant, and duration).[34]
4. Horses: Arterial (carotid) and capillary (skin) blood samples were collected from 12 horses that were either clinically healthy or had disorders that did not create significant acid-base abnormalities. The capillary blood was collected from a cutaneous incision after the area had been infused with a local anesthetic and rubbed with a histamine cream; the skin was not heated prior to sample collection. Significant differences in the arterial and capillary blood gas data (pH, pCO_2, HCO_3^-) were not found; pO_2 data were not reported.[35]

D. Heparinized whole blood
1. Blood may be collected in either glass or quality plastic syringes. To prevent exposure to air, the needle must be sealed with cork or rubber immediately after collection. Air should not be in the syringe during or after the collection of a sample.
2. Heparin is the preferred anticoagulant for combined blood gas/electrolyte samples, but excessive amounts of heparin can cause erroneous results (e.g., decreased [fCa^{2+}]) and inadequate amounts can increase the likelihood of a clotted sample. If using either dry-balanced or liquid heparin, the heparin concentration in the sample should be no more the 20 IU/mL.[1]
   a. Dry (lyophilized) heparin is preferred, especially if Na^+, K^+, Cl , and fCa^{2+} concentrations are going to be measured concurrently.[1] Commercial syringes are available that contain 7–40 IU of dry-balanced heparin. However, commercial dry-balanced syringes are not all the same; differences in fCa^{2+}, Na^+, and K^+ concentrations were found when samples were collected into syringes produced by three different companies.[36]
   b. If the only purpose of the sample is blood gas analysis (pH, pO_2, pCO_2), then liquid heparin is acceptable at higher concentrations if dilution is not excessive. For a 2 mL syringe in which heparin coats the walls and fills the dead space of the hub, about 0.1 mL of heparin (at 1000 IU/mL) in 2 mL of blood represents of 5 % dilution and final heparin concentration of 50 IU/mL of blood.
   c. Liquid heparin is reported to have a pH of approximately 6.4, pCO_2 near 7 mmHg (or less), and a pO_2 near 160 mmHg (at sea level), values very different from blood.[37] Thus for an animal breathing air, excess heparin in the sample can lower the sample's pH and pCO_2 while raising the sample's pO_2 (see Chapter 9 for effects on Na^+, K^+, and Cl^- concentrations; see Chapter 11 for effects on fCa^{2+} concentration).
   d. Larger volumes of heparin also decrease pCO_2(a), [HCO_3^-], [K^+], and lactate concentrations, and increase pO_2(a) (if breathing air) and [Na^+].[38] Statistically significant differences are seen with various amounts of heparin left in a syringe's dead space. Changes that were considered potentially clinically relevant were found when there was heparin at the 0.1 mL mark before blood was collected to the 1 mL volume.
3. Optimally, a heparinized blood sample should be tested (point-of-care testing) or transported to the laboratory immediately after collection.

a. When collected in a plastic syringe, the sample should be kept at room temperature and analyzed within 30 min.[1]
b. The plastic-syringe sample should not be placed in an ice bath; O_2 diffuses more quickly through polypropylene plastic when its temperature is lowered because the cold sample has a lower pO_2 (thus greater pressure gradient with air) and Hgb has greater O_2 affinity. Cold-induced changes in pO_2(a) and pCO_2(a) have been shown experimentally.[39,40] pCO_2 is minimally affected by cold temperature because the pCO_2-gradient is reduced in the cold.[39,40] As there are minimal changes in pCO_2, there will be minimal changes in $[HCO_3^-]$ and pH if sample analysis is not delayed.
c. If collected in a glass syringe, acceptable blood gas and pH results can be obtained if the heparinized blood is immersed in an ice bath and analyzed within 1 h. Placement of the sample in a refrigerator will not cool the sample as quickly; do not place a sample in a freezer, because RBCs will lyse.

4. Collecting blood into a heparin-containing (green-top) evacuated tube does not yield an acceptable sample.[41] Even when filled with an appropriate blood volume, pO_2 in the dead space is greater than blood pO_2(a) or pO_2(v) values (if animal is breathing ambient air) and the pCO_2 is much less than either pCO_2(a) or pCO_2(v) values. The loss of $CO_{2(g)}$ to the dead space can also be detected by a decrease in the $[HCO_3^-]$ or $[tCO_2]$ in clot tubes[42] (see Chapter 9 for effects on Na^+, K^+, and Cl^- concentrations; see Chapter 11 for effects on $[fCa^{2+}]$).

E. Erroneous blood gas and pH values because of poor sample collection or handling
1. Exposure to air (including air bubbles) or excess liquid heparin in the sample (the pO_2 and pCO_2 of liquid heparin is the same as room air if heparin is exposed to air)
a. Net result if exposed to air: ↑ pH, ↑ pO_2, ↓ pCO_2, and ↓ $[HCO_3^-]$ (heparin may lower the pH due to its acidity)
b. The pO_2 and pCO_2 values for arterial and venous blood versus room air are compared in Table 10.3.
c. The pO_2 and pCO_2 of blood and air quickly equilibrate; the blood pO_2 increases (unless the patient is on O_2 therapy) and blood pCO_2 decreases. The loss of CO_2 from blood shifts the equilibrium in Eqs. 10.2a and 10.2b to the right, causing decreases in the $[HCO_3^-]$ and $[H^+]$ of the sample.
d. When 55 μL of air was added to a 2 mL arterial blood sample, significant increases in pO_2(a) were found within 1 min, and decreases in pCO_2(a) occurred within 30 min when the sample was in a glass syringe placed in an ice bath.[44]

2. Delay in sample analysis (but no air exposure)
a. Net result: ↓ pH, ↓ pO_2, and ↑ pCO_2
b. Aerobic metabolism of leukocytes and platelets causes decreased pO_2. Metabolic processes in leukocytes, RBCs, and platelets produce H^+ and CO_2.
c. Based on stability studies for equine blood pO_2(a), arterial blood should be analyzed within 10 min (if kept at room temperature) or within 2 h (if using a glass syringe

Table 10.3. Differences in pO_2 and pCO_2 (mmHg) between air and blood (samples from a healthy animal inspiring room air)[43]

	Room air	Arterial blood	Venous blood
pO_2	≅150	≅90	≅40
pCO_2	< 1	≅40	≅46

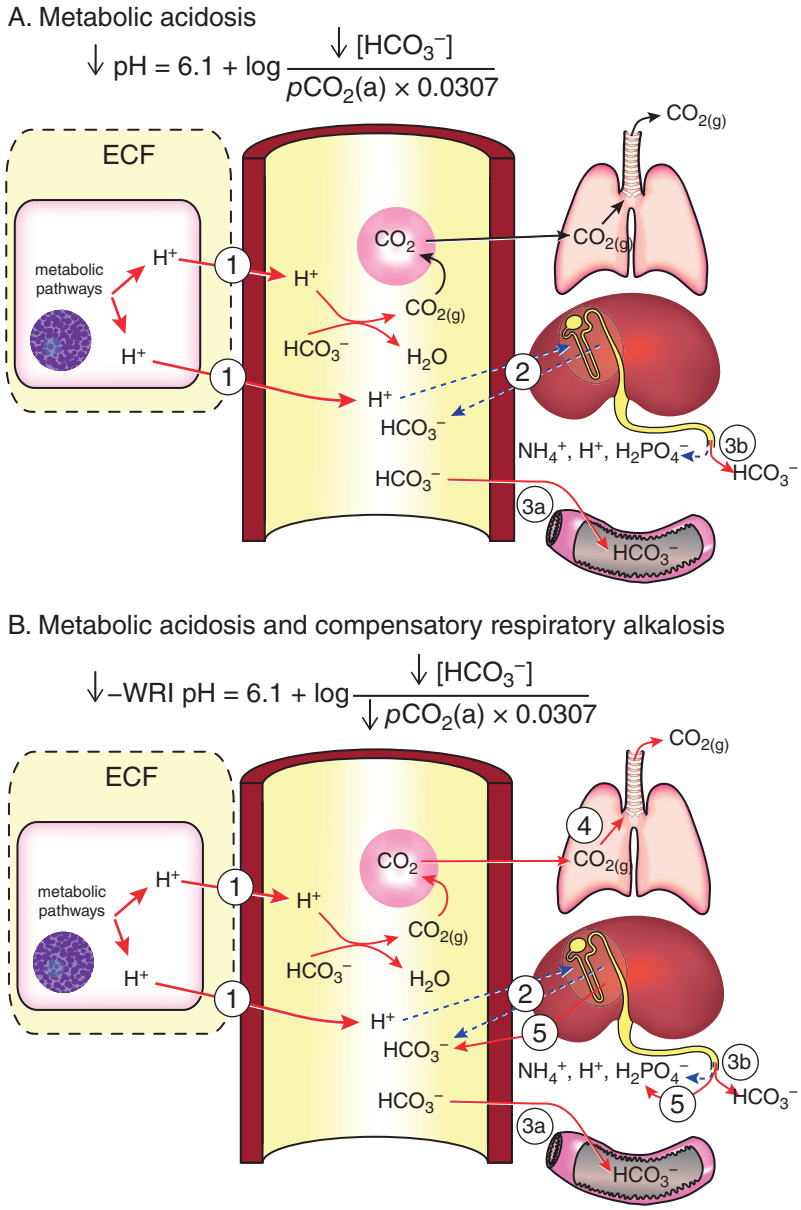


Figure 10.6. Metabolic acidosis. Effects on the Henderson–Hasselbalch equation are shown.

A. There are three major reasons for a metabolic acidosis and in each, H^+ tends to accumulate and thus acidemia may occur.

① Excess H^+ is being produced by metabolic pathways. The $[HCO_3^-]$ decreases as it buffers the generated H^+. The two most common forms of this acid-base disorder are lactic acidosis and ketoacidosis.

② There is decreased renal/urinary excretion of H^+. The $[HCO_3^-]$ decreases because it is being used to buffer the accumulating H^+. Acute and chronic renal failures are common causes of this type of acidosis; in these disorders, there also is decreased HCO_3^- formation. Other disorders in which there is decreased urinary excretion of H^+ include uroperitoneum, obstructive uropathy, and distal renal tubular acidosis.

③ In some forms of diarrhea (especially in horses), there is an increased intestinal loss of HCO_3^- that results in a decreased plasma $[HCO_3^-]$ (3a). Ruminant and camelid choke may also result in a decreased $[HCO_3^-]$

stored in an ice bath). The pCO_2(a) and pH were stable for up to 1 h at room temperature.[45]

d. When human arterial blood was collected in heparinized plastic syringes via an arterial line, pO_2 was significantly increased after 60 min at 0 °C but not at 22 °C storage. The pCO_2 was significantly increased and the pH was significantly decreased after 30 and 60 min at 22 °C but not 0 °C storage.[46] Changes were mild at 60 min and not tested beyond 60 min. (Note: See IV.D.3.b above for gas diffusion through plastic.)

ACID-BASE ABNORMALITIES (using traditional definitions from Henderson–Hasselbalch equation)

I. Metabolic (nonrespiratory) acidosis (Figure 10.6A)

A. *Metabolic acidosis* is a pathophysiologic state (condition) in which a nonrespiratory process promotes a decrease in plasma or serum [HCO_3^-] and an associated rise of [H^+] in plasma. When the state is the primary process, acidemia may develop. When the state is compensatory, the plasma pH reflects a shift away from alkalemia.

B. Metabolic acidoses are typically produced by the excess generation of H^+, decreased urinary excretion of H^+, or an excessive loss of HCO_3^-. The loss of HCO_3^- (or other body buffers) reduces the buffering capacity of the body and thus allows H^+ to accumulate. As seen in the Henderson–Hasselbalch equation (Eq. 10.1a), a decreased [HCO_3^-] without a change in the pCO_2 (arterial or venous) requires the pH to decrease. At times it is important to remember that H^+ does not accumulate in plasma as long as the body's buffers are handling either increased H^+ production or decreased H^+ excretion.

C. Disorders and pathogeneses (Table 9.10 and Figure 10.6A)

D. The metabolic acidoses are sometimes classified into one of five groups that are not mutually exclusive:

Figure 10.6. *continued*

because the animals do not swallow the HCO_3^--rich saliva (process not shown). In proximal renal tubular acidosis, there is increased urinary loss of HCO_3^- (3b). When the plasma [HCO_3^-] decreases because of either intestinal or renal loss and there is no change in the pCO_2(a), the plasma pH must decrease.

B. Respiratory or metabolic compensation to the acidemia. Effects on the Henderson–Hasselbalch equation are shown.

①②③ Same as for part A

④ The respiratory response to acidemia is hyperventilation, which increases excretion of CO_2 and concurrently removes H^+ from plasma as the equilibrium of the bicarbonate/carbonic acid pathway shifts toward CO_2 and H_2O. The low pCO_2(a) represents a compensatory respiratory alkalosis. Acidemia may still be present, or the plasma pH may increase enough to be WRI, but the pH will never be completely corrected as long as the primary metabolic problem is present.

⑤ If impaired urinary excretion of H^+ is not the cause of the metabolic acidosis (process ②), the renal response to acidemia is to increase excretion of H^+ and concurrently increase production of HCO_3^- (Figures 9.4 and 9.7). The resultant increase in [HCO_3^-] represents a compensatory metabolic alkalosis.

Solid red arrow – increased rate; *dashed blue arrow* – decreased rate

Note: Changes in venous [HCO_3^-] typically reflect changes in arterial [HCO_3^-]. Changes in pCO_2(v) may reflect pCO_2(a) changes but less reliably, especially with circulatory failure or during mechanical ventilation.

1. *Titrational acidosis*: [HCO_3^-] decreases because it is being used to buffer H^+ that is either being produced excessively (e.g., lactic acidosis or ketoacidosis) or not being adequately excreted by kidneys (e.g., renal failure). *Titrational* is somewhat of a misnomer because *titrating* is the chemistry laboratory process of adding a reagent to produce a given effect (e.g., adding acid to determine the concentration of a base). The decreased [HCO_3^-] in most metabolic acidoses is caused by buffering, not by titrating. HCO_3^- is one of several buffers in blood and is used to assess alterations in the concentrations of all buffers.
2. *Secretory (secretional) acidosis*: [HCO_3^-] decreases because it is being lost from the body (e.g., excess salivary loss or proximal renal tubular acidosis) or not being produced (distal renal tubular acidosis). The hallmark of these acidoses is the concurrent hyperchloremia. *Secretory* may be an appropriate adjective when HCO_3^- is being secreted (e.g., in saliva). However, in the context of the tubular acidoses, *secretory* is not an appropriate adjective because the decreased [HCO_3^-] is not caused by the secretion of HCO_3^- into the tubular fluid (Figures 9.4 and 9.8).
3. *Organic acidosis*: The decrease in [HCO_3^-] is linked to the accumulation of an organic anion (carbon-containing molecules such as lactate, AcAc, BHB, or citrate). The organic anion may be from an exogenous source, or it may represent the increased production of an organic anion by metabolic pathways (see later sections of lactic acidosis and ketoacidosis).
4. *Inorganic acidosis*: The decrease in [HCO_3^-] is linked to the accumulation of an inorganic anion (molecules such as PO_4 or sulfate that do not contain carbon). The metabolic acidosis of renal failure is sometimes called an inorganic acidosis (due to increased [PO_4]), but there also may be increased concentrations of organic anions (e.g., citrate).
5. *Dilutional acidosis*: This occurs from an excess of H_2O in plasma. When considered in terms of the SID approach to acid-base disorders (see the Strong-Ion Difference section), the excess of H_2O dilutes the electrolytes proportionately (e.g., decreases both [Na^+] and [Cl^-] by 20 %). However, a 20 % decrease in [Na^+] (e.g., from 150 mmol/L to 120 mmol/L) results in a greater incremental decrease than the 20 % decrease in [Cl^-] (e.g., from 110 mmol/L to 88 mmol/L, a 22 mmol/L rather than a 30 mmol/L decrement), and thus the SID decreases. When viewed from the traditional approach to acid-base disorders, the excess H_2O dilutes the HCO_3^- and thus causes a metabolic acidosis (e.g., a 20 % dilution would decrease [HCO_3^-] from 24.0 mmol/L to 19.2 mmol/L). Some people also consider the hyperchloremic acidosis that is caused by the rapid infusion of saline ([Na^+] and [Cl^-] = 154 mmol/L, but [HCO_3^-] $\cong$ 0 mmol/L) to be a dilutional acidosis. Saline is not solute-poor H_2O but does contain a higher percentage of H_2O (nearly 100 %) per unit volume than does plasma (about 93 %).

E. Physiologic compensation for metabolic acidosis (Figure 10.6B)
   1. Acidemia stimulates the central respiratory chemoreceptors to cause hyperventilation. Hyperventilation increases the excretion of CO_2, thus reducing the blood pCO_2 (arterial and venous) and [H^+] (compensatory respiratory alkalosis).
   2. In nonrenal disorders, the kidneys increase the secretion of H^+ (Figures 9.4 and 9.7).

F. Acidemia of lactic acidosis[47]
   1. As shown in Figure 9.11, a major product of anaerobic respiration is L-lactate, not L-lactic acid. Figure 9.11 also illustrates the concept that increased formation of H^+ is from ATP degradation in the absence of enough oxidative respiration to efficiently reuse the generated H^+. Concurrent generation of H^+ in other metabolic pathways during hypoxia likely contributes to the accumulation of H^+ in the blood (acidemia) (Figure 10.7).

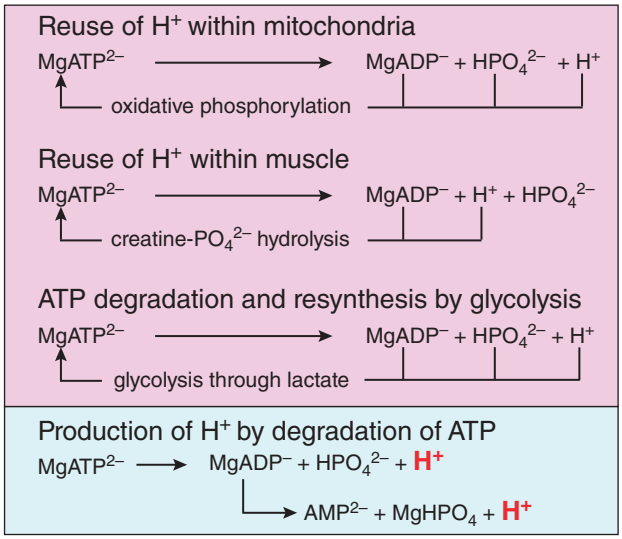


Figure 10.7. Acidemia of lactic acidosis. ATP is ionized in neutral solutions, so it exists mostly complexed with Mg^{2+} as $MgATP^{2-}$ in cells. The top three reactions show that H^+ released when ATP is dephosphorylated is then used in ATP-synthesis reactions or pyruvate reduction to lactate so that H^+ does not accumulate. The bottom reaction shows that H^+ released from ATP degradation may not be used in subsequent metabolic pathways and therefore may accumulate and cause acidemia (Figure 9.11).[47]

- Reuse of H^+ within mitochondria: When aerobic conditions are adequate, the H^+ released during the dephosphorylation of ATP is used during oxidative phosphorylation to produce more ATP.
- Reuse of H^+ within muscles: The H^+ released during the dephosphorylation of ATP is used in the creatine kinase reaction when creatine-PO_4 is converted to creatine and new ATP is synthesized.
- ATP degradation and resynthesis by glycolysis: The ADP and PO_4 from ATP degradation in the cytosol are used to form more ATP during glycolysis. The H^+ generated during ATP and pyruvate formation is used in the LD-catalyzed reduction of pyruvate to lactate; any H^+ not used would contribute to acidemia.
- Production of H^+ by degradation of ATP: For each ATP that is degraded to AMP, two H^+ are produced and they promote acidemia.

2. Concurrent triglyceride lipolysis stimulated by catecholamines can also generate H^+ ions (see the following ketoacidosis section).
3. The pK_a of lactic acid is 3.86, so at a plasma pH of 7.4, the ratio of L-lactate to L-lactic acid is > 1000.
4. Because lactate and not lactic acid accumulates and because the acidemia is from ATP hydrolysis and not lactic acid production, perhaps better classifications of the disorder would be lactate-associated acidosis or metabolic acidosis with hyperlactatemia, instead of lactic acidosis.

G. Acidemia of ketoacidosis

1. As described in Chapter 9, several disorders or conditions can create the negative energy balance in which excessive β-oxidation of fatty acids and the resulting excess formation of acetyl-CoA can promote ketogenesis to produce AcAc, BHB, and acetone. However, these molecules are not acids, and thus are not the source of the acidemia that commonly is found with ketosis. The reduction of AcAc to BHB actually consumes H^+.

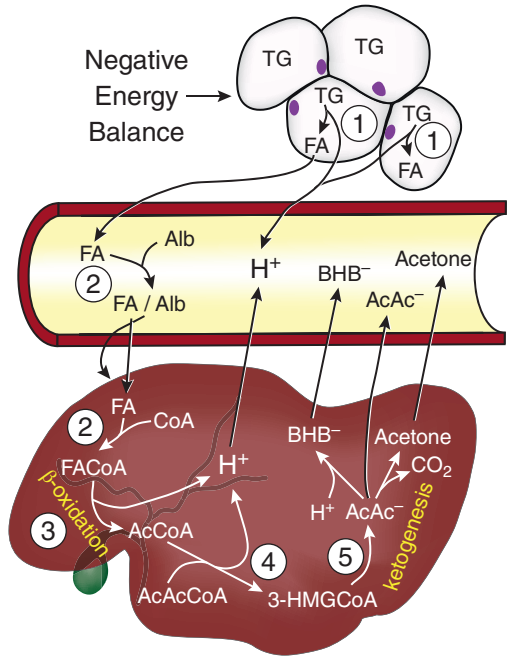


Figure 10.8. Acidemia of ketoacidosis. The acidemia of ketoacidosis is created by excessive H^+ release during lipolysis in adipocytes ①, increased β-oxidation of fatty acids in hepatocytes ③, and increased formation of 3-HMGCoA ④ in hepatocytes prior to ketogenesis.

① When an animal is in a negative-energy status, stimuli promote triglyceride (TG) lipolysis and the release of fatty acids (FA) and H^+.

② Fatty acids bind to plasma albumin (Alb) and the complexes are transported to the liver. Fatty acids enter hepatocytes in which they react with coenzyme-A (CoA) to form fatty acid-CoA derivatives (FACoA).

③ β-oxidation of a fatty acids produces acetyl-CoA (AcCoA) and releases H^+.

④ Two AcCoA molecules combine to form acetoacetyl-CoA (AcAcCoA; not shown). That molecule condenses with another AcCoA to form 3-HMGCoA, releasing H^+.

⑤ Breakdown of 3-HMGCoA leads to formation of acetoacetate ($AcAc^-$) which can be converted to either acetone or β-hydroxybutryate (BHB^-). These three molecules are "ketone bodies" (BHB is not a ketone). Release of acetone and $AcAc^-$ produce ketonemia.

3-HMGCoA, 3-hydroxy-β-methylglutaryl-CoA

2. When a negative energy balance is the reason for ketosis, the source of the ketone bodies begins with the lipolysis of triglycerides in adipose tissue and the excessive release of H^+ occurs before ketogenesis (Figure 10.8).[48] Because ketone bodies and not ketoacids accumulate, and because the acidemia is from H^+ degradation of lipids and not ketoacid production, perhaps a better classification of the disorder would be ketone-associated acidosis instead of ketoacidosis.
3. Acetic acid's pK_a is 3.58 and β-hydroxybutyric acid's pK_a is 4.70, so at a plasma pH of 7.4, the ratios of anions to acids are > 1000 and > 100, respectively.

II. Respiratory acidosis

A. *Respiratory acidosis* is a pathophysiologic state (condition) in which a respiratory disorder or secondary response promotes an increase in plasma pCO_2 (arterial and probably venous)

(hypercapnia when pCO_2 is increased), and an associated accumulation of H^+ in plasma. Metabolism continues to generate H^+, but the animal cannot remove it adequately via the bicarbonate buffering system (Figure 10.9A) or other physiologic processes. Note that increased pCO_2(v) (including central venous pCO_2) may reflect circulatory failure and poor perfusion rather than respiratory acidosis.

B. Hypoxemia accompanies (and typically precedes) hypercapnia if patients are breathing room air.

C. Disorders or conditions (Table 10.4)

D. Physiologic compensation for respiratory acidosis (Figure 10.9B): The renal response is detectable within a few hours and takes about 2–5 d for the blood pH to peak or plateau during chronic hypercapnia.[51,52]

III. Metabolic (nonrespiratory) alkalosis

A. *Metabolic alkalosis* is a pathophysiologic state (condition) in which a nonrespiratory process promotes an increase in serum and plasma [HCO_3^-] and an associated depletion of H^+ from plasma. When the state is the primary process, alkalemia may develop. When the state is compensatory, the plasma pH reflects a shift away from acidemia.

B. Metabolic alkaloses are typically produced through the generation of HCO_3^- by renal tubules or gastric/abomasal mucosa when accompanied by loss of H^+ via renal or gastric/abomasal secretions (Figure 10.10). The production of HCO_3^- and concurrent loss of H^+ are illustrated in Figure 9.4 (type A intercalated renal tubular cells) and Figure 9.6 (gastric parietal epithelial cell). Note that the H^+ secreted by the gastric mucosa and type A intercalated cells is produced in the epithelial cells; it is not lost from plasma.

C. Disorders and pathogeneses (Figure 10.10A, Table 9.9, and the associated Chapter 9 text)

D. Physiologic response (Figure 10.10B)

1. In nonrenal disorders associated with metabolic alkalosis, the kidneys are expected to decrease the secretion of H^+ and conserve less HCO_3^- (exception: see "paradoxical" aciduria below, Section III.D.3).
   a. With increased plasma [HCO_3^-], the amount of HCO_3^- that enters the filtrate may exceed the capacity of the PCTs to conserve it.
   b. Excess HCO_3^- is secreted by intercalated cells (type B) of the cortical collecting ducts (Figure 9.9).
2. Alkalemia inhibits the central respiratory chemoreceptors and thus causes hypoventilation. Hypoventilation decreases the pulmonary excretion of CO_2, thus elevating the plasma pCO_2(a) and increasing the [H^+] (compensatory respiratory acidosis) (Figure 10.10B).
3. *"Paradoxical" aciduria*
   a. It is called *paradoxical* because the aciduria is concurrent with alkalosis, but when the mechanisms are understood, the aciduria is not a paradox and thus becomes a nonparadoxical aciduria.
   b. When an animal has concurrent metabolic alkalosis, hypochloremia, and hypovolemia with or without hypokalemia, the kidneys may produce acidic urine. This is seen most frequently in cattle but also occurs in other mammals.
      (1) Hypochloremia is the most important contributor to the aciduria. Decreased filtrate Cl^- enhances renal H^+ secretion and HCO_3^- conservation by Na^+-dependent and Na^+-independent mechanisms.
         (a) Na^+-dependent: Decreased filtrate [Cl^-] limits Na^+ resorption in the PCT (electroneutrality) because Cl^- is the only quantitatively important

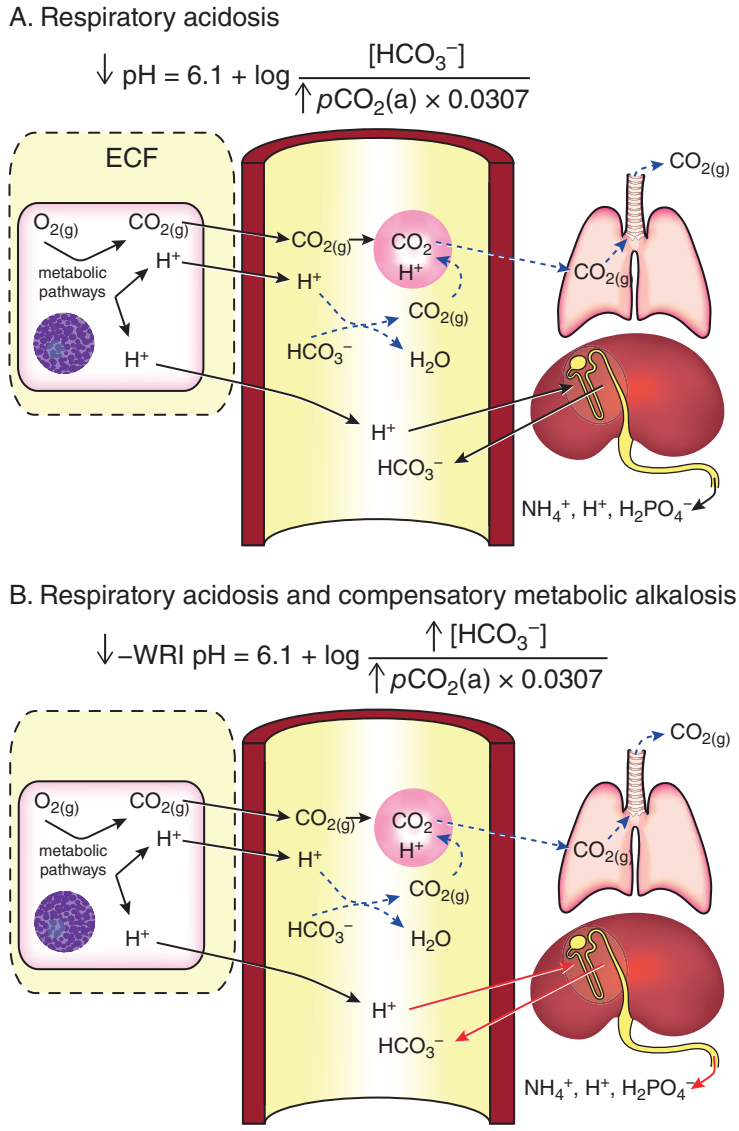


Figure 10.9. Respiratory acidosis. Effects on the Henderson–Hasselbalch equation are shown.

A. Respiratory acidosis occurs when there is decreased removal of CO_2 from plasma, thus increasing pCO_2(a). Consequently, there is less removal of H^+ from plasma via the bicarbonate/carbonic acid pathway, so acidemia may develop. The hypoventilation can occur because of pulmonary disease and other factors that reduce pulmonary function (Table 10.4). When the plasma pCO_2(a) increases and there is no change in the plasma [HCO_3^-], the plasma pH must decrease.

B. The renal responses to acidemia are to increase excretion of H^+ and concurrently increase production of HCO_3^- (Figures 9.4 and 9.7). The greater [HCO_3^-] represents compensatory metabolic alkalosis. Acidemia may still be present, or the plasma pH may increase enough to be WRI, but the pH will never be completely corrected as long as the primary respiratory problem is present.

Red solid arrow – increased rate; *blue dashed arrow* – decreased rate

Note: Changes in venous [HCO_3^-] typically reflect changes in arterial [HCO_3^-]. Changes in pCO_2(v) may reflect pCO_2(a) changes but less reliably, especially with circulatory failure or during mechanical ventilation.

Table 10.4. Disorders and conditions that cause respiratory acidosis (hypoventilation)[a]
Inhibition or dysfunction of medullary respiratory center
Drugs:* anesthetics, sedatives, and narcotics
Brain stem disease (trauma, infection, neoplasia, etc.)
Alkalemia due to metabolic alkalosis
Inhibition or dysfunction of respiratory muscles (diaphragm, chest wall): cervical spinal cord injury, muscle fatigue, tick paralysis, tetanus, botulism, myasthenia gravis, hypokalemia, and succinylcholine
Upper airway dysfunction: foreign body, vomitus, and mechanical hypoventilation
Impaired gas exchange at pulmonary capillaries
Pulmonary disease:* infection, allergy, edema (could be secondary to cardiac failure), fibrosis, neoplasms, emphysema, atelectasis, and hyaline membrane disease (neonates)
Restrictive disease:* pneumothorax, pleural effusions, diaphragmatic hernia, abdominal distension, and thoracic wall fractures
Vascular disorders: right-to-left shunts (e.g., patent ductus arteriosus)

* A relatively common disorder, condition, or mechanism

[a] Disorders that cause respiratory acidosis – increased $pCO_2(a)$ – because of reduced gas exchange in the lungs will likely also result in decreased $pO_2(a)$, but the decrease may not be sufficient for the disorder to be classified as a hypoxemic disorder (Table 10.9).

Note: During their first few days of life, neonatal foals and calves may have greater $pCO_2(a)$ than adults.[49,50]

resorbable anion in the filtrate. Na^+ resorption distally must therefore occur with H^+ or K^+ excretion to maintain electroneutrality, thus promoting aciduria and hypokalemia (Figure 9.2).

(b) Na^+-independent: Hypochloremia decreases type B intercalated cell HCO_3^- secretion because decreased luminal $[Cl^-]$ limits luminal Cl^-/HCO_3^- exchange (Figure 9.9). Also, hypochloremia is sensed by the macula densa, thus stimulating the renin–angiotensin–aldosterone system, which increases distal H^+ secretion from type A intercalated cells (Figure 9.4). Decreased filtrate $[Cl^-]$ creates a Cl^- gradient that probably increases Cl^- cosecretion (to maintain electroneutrality) and HCO_3^- generation.

(2) Hypovolemia also promotes renal HCO_3^- conservation and increased urinary excretion of H^+ through activation of the renin–angiotensin–aldosterone system. This helps maintain blood volume but exacerbates the metabolic alkalosis and alkalemia.

(a) Aldosterone increases H^+ secretion from type A intercalated cells, thus promoting HCO_3^- formation and conservation (Figure 9.4).

(b) Aldosterone also promotes H^+ secretion from collecting duct principal cells via Na^+ resorption, which creates a negative charge potential in the lumen, thus reducing back-diffusion of H^+ (Figure 9.2). K^+ secretion and hypokalemia are also promoted. Lastly, less luminal Cl^- in the collecting duct (see above explanations) limits the peritubular Cl^- resorption and thus limits correction of the negative charge potential, further reducing back-diffusion of H^+.

(3) Hypokalemia: Renal tubular cell K^+/H^+ shifts promote H^+ secretion and HCO_3^- conservation through the luminal Na^+-H^+ antiporter in the PCT and through stimulation of the H^+-K^+-ATPase pump to resorb K^+ and secrete H^+ in the distal convoluted tubule.

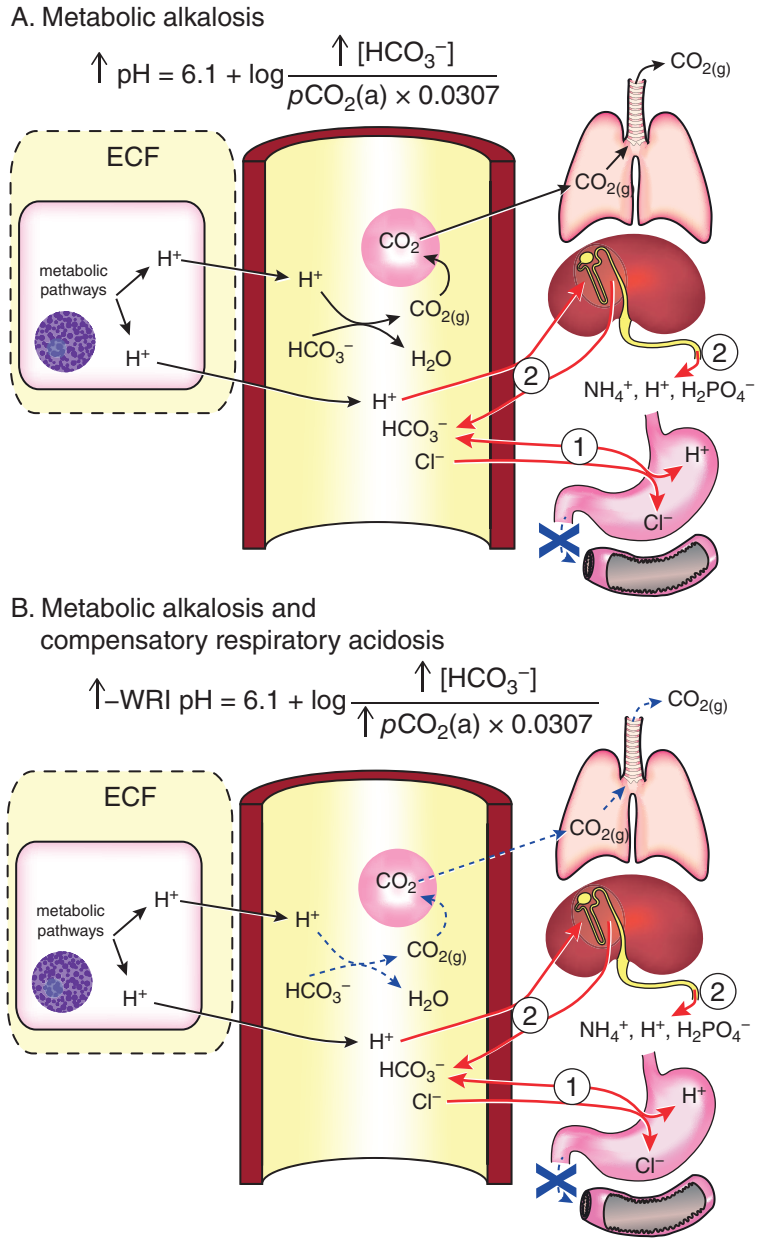


Figure 10.10. Metabolic alkalosis. Effects on the Henderson–Hasselbalch equation are shown.

A. There are two major reasons for a primary metabolic alkalosis, and in each, $[H^+]$ tends to decrease and thus alkalemia may occur.

① The gastric or abomasal secretion of H^+ and Cl^- results in the production of HCO_3^- that enters plasma (Figure 9.6). When persistent vomiting (dogs and cats) or when an obstruction prevents abomasal (ruminant) or forestomach (camelid) fluids from entering the intestine, the HCO_3^- accumulates in plasma because it is not used to buffer the H^+ that is normally absorbed in the intestine.

② Increased renal excretion of H^+ causes an increased renal production of HCO_3^- that can increase plasma $[HCO_3^-]$. This form of metabolic alkalosis can occur with increased aldosterone activity (Figures 9.2 and 9.4),

IV. Respiratory alkalosis

A. *Respiratory alkalosis* is a pathophysiologic state (condition) in which a respiratory disorder or secondary response promotes a decrease in plasma pCO_2 (arterial and probably venous) (hypocapnia when pCO_2 is decreased) and an associated depletion of H^+ in plasma. When the state is the primary process, alkalemia may develop. When the state is compensatory, the plasma pH reflects a shift away from acidemia.

B. Respiratory alkaloses occur because of alveolar hyperventilation; respirations remove CO_2 and thus H^+ at a rate faster than H^+ is being made (Figure 10.11A).

C. Disorders or conditions (Table 10.5)

1. Hypoxemia disorders (Table 10.9): Peripheral chemoreceptors (carotid and aortic bodies) stimulated by hypoxemia initiate hyperventilation that causes the increased pulmonary loss of CO_2 and thus loss of plasma H^+.
   a. Because CO_2 diffuses more easily than O_2, hyperventilation may decrease pCO_2(a) while the animal remains hypoxemic.
   b. If there is acute hypoxemia, loss of CO_2 may be somewhat self-limiting because the resultant alkalemia inhibits respiration. However, with chronic hypoxemia and respiratory alkalosis, the resulting compensatory metabolic acidosis reduces the degree of alkalemia, and thus hypoxemia continues to stimulate hyperventilation.[53]
   c. Pulmonary disease that impairs gas exchange and results in hypoxemia may cause alveolar hypoventilation and thus may reduce loss of CO_2, even enough to increase pCO_2(a) (respiratory acidosis).
2. Stimulation of the respiratory center: Acidemia and other factors, including central nervous system disease, may stimulate the central respiratory center to cause hyperventilation and thus a loss of CO_2 and H^+. If moderate to marked anemia causes respiratory alkalosis, it might be secondary to hypoxia of central neural tissue with subsequent local decreases in pH that stimulate central chemoreceptors;[54] although anemia is often lumped with causes of hypoxemia, blood O_2ct is typically decreased without hypoxemia (decreased arterial pO_2), the major stimulus for peripheral chemoreceptors.
3. Mechanical hyperventilation: Animals assisted by mechanical ventilators may develop alkalemia if there is excessive loss of CO_2.
4. Pain or anxiety elicit neurologic responses that increase frequency or depth of breathing and therefore increase ventilation.

Figure 10.10. *continued*

with marked hypokalemia, and with administration of loop diuretics. When an animal is hypovolemic, hypochloremic, and hypokalemic (as frequently occurs with gastric/abomasal outflow disorders), functional kidneys produce HCO_3^- (contributes to alkalosis) and increase the renal excretion of H^+ ("paradoxical" aciduria).

When the plasma [HCO_3^-] increases and there is no change in the pCO_2(a), the plasma pH must increase.

B. The respiratory response to alkalemia is hypoventilation to decrease excretion of CO_2, and thus there is less removal of H^+ from plasma. The higher pCO_2(a) represents a compensatory respiratory acidosis. Alkalemia may still be present, or the plasma pH may decrease enough to be WRI, but the pH will never be completely corrected as long as the primary metabolic problem is present.

Red solid arrow – increased rate; *blue dashed arrow* – decreased rate

Note: Changes in venous [HCO_3^-] typically reflect changes in arterial [HCO_3^-]. Changes in pCO_2(v) may reflect pCO_2(a) but less reliably, especially with circulatory failure or during mechanical ventilation.

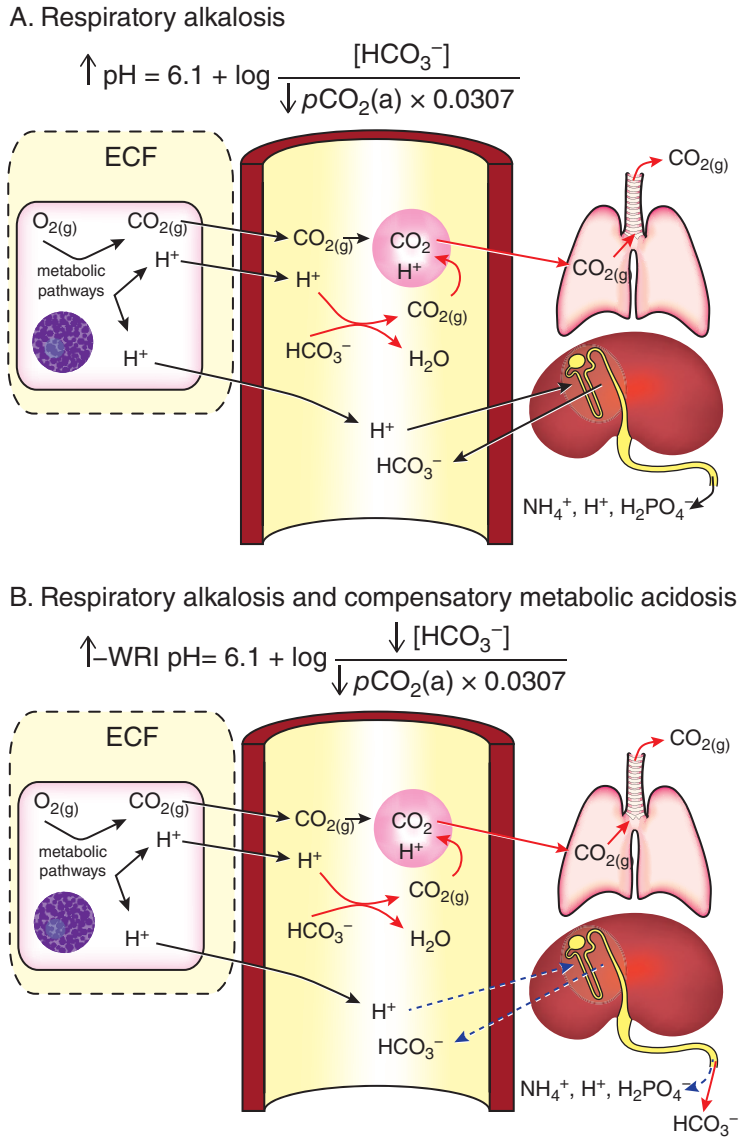


Figure 10.11. Respiratory alkalosis. Effects on the Henderson–Hasselbalch equation are shown.

A. Respiratory alkalosis occurs when there is increased removal of CO_2 from plasma. Because there is consequently increased removal of H^+ from plasma via the bicarbonate/carbonic acid pathway, alkalemia may develop. The hyperventilation can occur because of factors that stimulate respiration (Table 10.5). When the plasma pCO_2(a) decreases and there is no change in the plasma [HCO_3^-], the plasma pH must increase. The decrease in [H^+] is not matched by a detectable decrease in [HCO_3^-] because at a pH of 7.4, the [H^+] is near 40 nmol/L whereas the [HCO_3^-] is near 24 mmol/L (600 times the [H^+]).

B. The renal response to alkalemia is to decrease excretion of H^+ (which also decreases production of HCO_3^-) and increase excretion of HCO_3^-. The lower [HCO_3^-] represents a compensatory metabolic acidosis. Alkalemia may still be present, or the plasma pH may decrease enough to be WRI, but the pH will never be completely corrected as long as the primary respiratory problem is present.

Red solid arrow – increased rate; *blue dashed arrow* – decreased rate

Note: Changes in venous [HCO_3^-] typically reflect changes in arterial [HCO_3^-]. Changes in pCO_2(v) may reflect pCO_2(a) but less reliably, especially with circulatory failure or during mechanical ventilation.

Table 10.5. Disorders and conditions that cause respiratory alkalosis (hyperventilation)
Hypoxemia: stimulation of peripheral chemoreceptors[a] (Table 10.9)
Stimulation of the respiratory center
Metabolic acidosis*
Septicemia (Gram negative)
Heat stroke or fever
Drugs: salicylates, aminophylline (theophylline)
Central neurologic disease: trauma, neoplasms, inflammation, cerebrovascular accident, hepatic encephalopathy, and possibly moderate to marked anemia (hypoxia)
Mechanical hyperventilation
Pain or anxiety

* A relatively common disorder, condition, or mechanism

[a] If hypoxemia stimulates the peripheral chemoreceptors (carotid or aortic bodies), increased respirations result in hyperventilation and thus decreased pCO_2(a) (respiratory alkalosis) if the CO_2 is actually exhaled.

Note: When determined by the i-STAT EC8+ cartridge, pCO_2 can be falsely decreased by the presence of thiopental in the blood sample.

5. Interferent: When determined by the i-STAT EC8+ cartridge, the pCO_2 can be falsely decreased by the presence of thiopental in the blood sample.[55] Also, i-STAT cartridge information indicates that the cartridge should not be used when propofol is in the sample, but the type and direction of interference were not stated.

D. Physiologic compensation
1. In response to alkalemia, healthy kidneys decrease the secretion of H^+ and, in doing so, reduce the conservation of HCO_3^- (compensatory metabolic acidosis) (Figure 10.11B). Also, alkalemia stimulates type B intercalated cells to secrete HCO_3^- (Figure 9.9).[53]
2. Decreases in pH can be detected within a few hours, but the renal response takes about 2–5 d for the increase in $[H^+]$ to plateau.

V. Classification of acid-base disorders (using traditional acid-base definitions)
A. Simple acid-base disorders are those in which the pathophysiologic state is limited to a primary disturbance (e.g., lactic acidosis) and the expected compensatory process (e.g., hyperventilation). The major patterns for the simple acid-base disorders are listed in Table 10.6. Figure 10.12 provides an algorithm for the classification of simple acid-base disorders.
B. When two or more simple acid-base disorders are present (e.g., vomiting causing metabolic alkalosis and renal failure causing metabolic acidosis), there is a *mixed acid-base disorder*. A compensatory or physiologic response to a primary acid-base disorder is not a mixed acid-base disorder.
1. Interpretation of acid-base data is improved when it is recognized that more than one pathologic state is contributing to the abnormalities.
2. It is essential that sample collection, processing, and analysis be done correctly so that the laboratory data are valid. Erroneous measured data produce erroneous calculated values and incorrect classifications.

VI. Mixed acid-base disorders: The following sections provide methods of approaching mixed acid-base disorders.

Table 10.6. Blood gas data in simple acid-base disorders

Disorder	pH	pCO_2(a)[a]	[HCO_3^-]
Respiratory acidosis	↓	↑	WRI
Respiratory acidosis + compensatory metabolic alkalosis	↓–WRI	↑	↑
Metabolic acidosis	↓	WRI	↓
Metabolic acidosis + compensatory respiratory alkalosis	↓–WRI	↓	↓
Respiratory alkalosis	↑	↓	WRI
Respiratory alkalosis + compensatory metabolic acidosis	↑–WRI	↓	↓
Metabolic alkalosis	↑	WRI	↑
Metabolic alkalosis + compensatory respiratory acidosis	↑–WRI	↑	↑

[a] Changes in pCO_2(v) may reflect pCO_2(a) but less reliably, especially with circulatory failure or during mechanical ventilation.

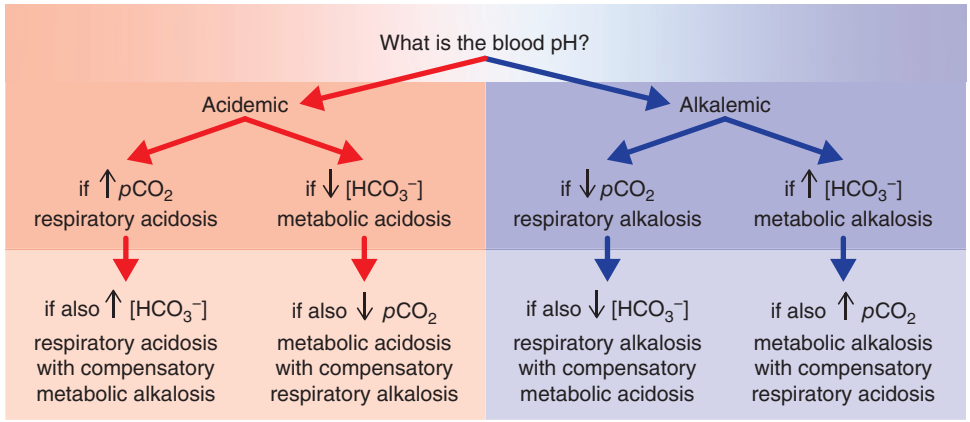


Figure 10.12. Algorithmic approach to classification of simple (not mixed) acid-base disorders. Refer to Tables 10.7 and 10.8 for expected compensatory responses.

A. Clinical clues for mixed acid-base disorders: The combination of history, a complete physical examination, and knowledge of the pathogeneses of simple acid-base disorders allow a veterinarian to recognize or suspect mixed acid-base disorders.
   1. If a dog has diabetes mellitus, ketonuria, and is vomiting, the dog very likely has both a metabolic acidosis (associated with ketosis and lipolysis) and a metabolic alkalosis (due to vomiting). Likewise, a dog that has chronic renal failure, and more recently has had persistent vomiting, likely has a metabolic acidosis and a metabolic alkalosis.
   2. If a dog with ketoacidotic diabetes mellitus is given fluids containing L-lactate, the dog would have a metabolic acidosis and an iatrogenic metabolic alkalosis after L-lactate metabolism leads to production of HCO_3^- (OH^- left after consumption of H^+ during lactate metabolism combines with CO_2 to form HCO_3^-).
   3. A dog with congestive heart failure causing edema may have been given large doses of furosemide. Poor oxygenation of blood may have caused hyperventilation (thus

respiratory alkalosis), and the excessive loop diuretic could cause iatrogenic metabolic alkalosis (see Chapter 9, Bicarbonate (HCO_3^-) and Total Carbon Dioxide (tCO_2) Concentrations, III.B.1.b.(1)).

B. Unexpected patterns in basic acid-base data
   1. An unexpected plasma pH (WRI or opposite expected change) for a given clinical state suggests a mixed acid-base disorder.
   2. pCO_2 (arterial or venous) and $[HCO_3^-]$ changes are in opposite directions[56]
      a. ↓ $[HCO_3^-]$ and ↑ pCO_2(a) would indicate both metabolic and respiratory acidoses as may occur with severe renal failure associated with hypercapnic respiratory failure or untreated cardiopulmonary arrest. ↑ pCO_2(v) would be supportive of respiratory acidosis.
      b. ↑ $[HCO_3^-]$ and ↓ pCO_2(a) would indicate both metabolic and respiratory alkaloses as may occur in congestive heart failure and excessive diuretic therapy. ↓ pCO_2(v) would be supportive of respiratory alkalosis.

C. Delta gap (Δ gap)[57]
   1. The Δ gap can be calculated (Eq. 10.8a) for a high-AG metabolic acidosis to see if the increase in AG matches the decrease in $[HCO_3^-]$. If not, it supports a mixed acid-base disorder. The upper limit of the AG RI and the lower limit of the $[HCO_3^-]$ RI are used for the calculation, and interpretation should include the analytical variance of the measured electrolyte concentrations.[57] A more complete description of using Δ gap is available.[58]

$$\Delta \text{ gap} = \Delta AG - \Delta HCO_3^-$$
$$\Delta AG = AG_{\text{patient}} - \text{AG upper reference limit} \qquad (\textbf{10.8a})$$
$$\Delta HCO_3^- = [HCO_3^-] \text{ lower reference limit} - [HCO_3^-]_{\text{patient}}$$

$$\Delta AG : \Delta HCO_3^- = \frac{\Delta AG}{\Delta HCO_3^-} = \frac{AG_{\text{patient}} - AG_{\text{middle of RI}}}{[HCO_3^-]_{\text{middle of RI}} - [HCO_3^-]_{\text{patient}}} \qquad (\textbf{10.8b})$$

   2. Using the Δ gap to interpret acid-base data requires valid RIs that apply to the patient's data; analytical differences produce different RIs in different laboratories. Also, patient data need to be free from preanalytical or analytical errors; sample exposure to air must be minimized so that the $[HCO_3^-]$ is valid.
   3. General concept for Δ gap: The calculated value is typically used to indicate how much of an increased AG is due to decreased $[HCO_3^-]$. The calculated value frequently can be predicted by quickly assessing the raw data to see if the common relationships of Na^+, Cl^-, and HCO_3^- concentrations are present: Are changes in $[Na^+]$ and $[Cl^-]$ proportional and is a decreased $[HCO_3^-]$ proportional to an increased $[Cl^-]$?
   4. Interpretation of Δ gap with increased AG[57]
      a. As originally described, a negative Δ gap was defined as < –6 mmol/L and a positive Δ gap was defined as > 6 mmol/L. This reflected the mean Δ gap of 0 ± 2SD as determined from the sum of the variances of $[Na^+]$, $[Cl^-]$, and $[HCO_3^-]$ in a particular laboratory using particular assays; appropriate cutoffs may vary with species and laboratories, so minor changes should be interpreted with caution and in light of other findings.
      b. If the Δ gap is near 0, then the AG increase is appropriately matched by a 1:1 decrease in $[HCO_3^-]$ as would occur with a simple high-AG metabolic acidosis (e.g., early lactic acidosis and ketoacidosis).

c. If the Δ gap is decreased (significantly negative), thus the [HCO_3^-] decreased more than the AG increased, then there is a mixed acid-base disorder with either a normal-AG acidosis in addition to the high-AG acidosis (e.g., lactic acidosis and acidosis of diarrhea) or a respiratory alkalosis with compensating hyperchloremic acidosis in addition to the high-AG acidosis (e.g., sepsis causing lactic acidosis and pulmonary edema). Preanalytical or analytical errors resulting in incorrect concentrations of Na^+, K^+, Cl^-, or HCO_3^- should also be considered.

d. If the Δ gap is increased (significantly positive), thus the [HCO_3^-] decreased less than the AG increased, then there is a mixed acid-base disorder with an alkalosis present in addition to the high-AG acidosis . This is usually a primary metabolic alkalosis in addition to the high-AG metabolic acidosis (e.g., persistent vomiting plus renal failure), but the patient could have respiratory acidosis with a compensatory metabolic alkalosis in addition to the high-AG metabolic acidosis. Preanalytical or analytical errors resulting in incorrect concentrations of Na^+, K^+, Cl^-, or HCO_3^- should also be considered.

D. Delta anion gap:Delta bicarbonate ratio ($\Delta AG:\Delta HCO_3^-$) (also called the gap-gap ratio)[59–61]

1. Similar to the Δ gap (previous section), the $\Delta AG:\Delta HCO_3^-$ has been calculated (Eq. 10.8b) to help recognize the presence of a mixed acid-base disorder when a high-AG metabolic acidosis is present. As for the Δ gap, valid RIs and patient data are needed for a useful calculated value.
2. The theory of the $\Delta AG:\Delta HCO_3^-$ is basically the same as that of the Δ gap except the changes in AG versus changes in [HCO_3^-] are expressed as a ratio instead of as a difference. With a simple high-AG metabolic acidosis, the $\Delta AG:\Delta HCO_3^-$ is close to 1.0.
   a. When there is a high-AG metabolic acidosis and there is a greatly increased $\Delta AG:\Delta HCO_3^-$ (e.g., near 2 or greater), then there is combination of high-AG metabolic acidosis and a metabolic alkalosis, e.g., renal failure plus vomiting or diabetic ketoacidosis plus vomiting.
   b. When there is a high-AG metabolic acidosis and there is clearly a decreased $\Delta AG:\Delta HCO_3^-$ (< 1), then there is evidence of a concurrent nonAG metabolic acidosis, e.g., ketoacidosis causing high-AG metabolic acidosis and diarrhea causing nonAG metabolic acidosis (hyperchloremic metabolic acidosis). Note: If the $\Delta AG:\Delta HCO_3^-$ < 1 but the AG is not increased, then there is a hyperchloremic metabolic acidosis, a state that may develop from proximal and distal renal tubular acidosis or some diarrheas (see Chapter 9).
   c. Several examples are described in the cited articles by Kraut and Goodkin.

E. Expected compensatory changes in acid-base disorders

1. Compensation for respiratory acidosis or alkalosis: If there is not renal disease, the kidneys are expected to alter their excretions of H^+ and HCO_3^- (also other electrolytes) to compensate for the acidemia or alkalemia.
2. Compensation for metabolic acidosis or alkalosis: If there is not renal disease and the acid-base disturbance is not of renal origin, then the kidneys are expected to alter their excretions of H^+ and HCO_3^- accordingly to compensate for the acidemia or alkalemia. The respiratory system is also expected to alter its excretion of CO_2 to compensate appropriately for acidemia or alkalemia.
3. When there is either respiratory or metabolic compensation, there is never complete compensation. Plasma pH may return to the RI, but it will never completely correct as long as the disorder causing the acid-base imbalance remains.

4. How much can these systems compensate if given time to do so? One aspect of the interpretation of blood gas data is attempting to determine whether the animal's respiratory or metabolic systems have compensated appropriately. If the data indicate that an animal has not achieved expected compensation, they may indicate the following:
   a. The animal has not had time to compensate. This is especially true of renal compensation for acute respiratory disorders as it takes several days to achieve appropriate renal compensation.
   b. The animal has more than one disorder that is altering the acid-base status (i.e., a mixed acid-base disorder), and this is preventing expected compensation.
5. To attempt to determine expected compensations in dogs, experimental acid-base disorders were created, and changes were monitored. The findings from several experiments were summarized in a review article;[62] data were extracted from that article for Table 10.7. The proposed purpose of the correction factors is to determine whether the changes in $[HCO_3^-]$ or pCO_2(a) represent a physiologic compensation to a single acid-base disorder or whether other factors are affecting the $[HCO_3^-]$ or pCO_2(a). The correction factors should be applied cautiously for several reasons.[63]
   a. The factors were derived via experimental conditions using healthy dogs. A dog's illness may involve processes that alter the responses. Correction factors for cats, horses, and cattle are not established.
   b. The factors were derived using arterial blood samples; venous blood does not have the same blood gas values and thus probably will not have the same correction factors. Correction factors for venous blood are not established.

Table 10.7. Expected compensations for acid-base disorders in dogs

Disorder	Expected $[HCO_3^-]$ change (mmol/L)[a,b]	Expected pCO_2(a) change (mmHg)[a,c]
For each mmHg ↑ in pCO_2(a) in:		
Acute respiratory acidosis (< 24 h)	↑ 0.15 (0.04–0.20)	—
Chronic respiratory acidosis (> 3–5 d)	↑ 0.35 (0.29–0.43)	—
For each mmHg ↓ in pCO_2(a) in:		
Acute respiratory alkalosis	↓ 0.25 (0.14–0.36)	—
Chronic respiratory alkalosis	↓ 0.55 (0.43–0.76)	—
For each mmol/L ↓ in $[HCO_3^-]$ in:		
Metabolic acidosis	—	↓ 0.7 (0.5–1.1)
For each mmol/L ↑ in $[HCO_3^-]$ in:		
Metabolic alkalosis	—	↑ 0.7 (0.5–1.3)

[a] Expected compensations were reported in a review article.[62] The first number is the recommended guideline value. The numbers in parentheses represent the interval of values reported in various experimental conditions that produced the acid-base disorders.

[b] HCO_3^- methods (either measured or calculated from blood gas data) are not precise enough to justify reporting $[HCO_3^-]$ to the nearest hundredth but may be precise enough to justify reporting concentrations to the nearest tenth. Therefore, instead of the reported expected changes of ↑ 0.15 (0.04–0.20), consideration of significant digits should result in an expected change of ↑ 0.2 (0.0–0.2).

[c] pCO_2(a) methods are not precise enough to justify reporting them to the nearest tenth but may be precise enough to justify reporting concentrations to the whole number. Therefore, instead of expected changes of ↓ 0.7 (0.5–1.1), consideration of significant digits should result in an expected change of ↓ 1 (0–1).

c. The calculated values can only be valid if the measured values are correct; preanalytical and analytical errors should be prevented.
d. As shown in Table 10.7, there was a range of expected [HCO_3^-] for each mmHg change in pCO_2(a). There also was a range of expected pCO_2(a) change for each mmol/L change in [HCO_3^-]. Using average values to calculate the expected change does not include biological variation in dogs (i.e., inter-individual differences) and the analytical variation in blood gas methods. The average values may or may not be appropriate for a given patient. Table 10.8 contains examples of the different conclusions for the same blood gas data if the average correction factor is used, if a higher correction factor (but one also found in healthy dogs) is used, and if different "health" values are used for [HCO_3^-] and pCO_2(a).
e. Not all recommendations for expected changes have specified if the pCO_2(a) values should be the measured values (37 °C for most blood gas instruments) or values corrected for the dog's body temperature (Table 10.2). For most of the cited studies in the review article,[62] the pCO_2(a) values were measured at 37 °C and then corrected for body temperature. Other cited articles did not state if the pCO_2(a) values were temperature corrected.

Table 10.8. Examples of expected compensation calculations for metabolic acidosis in dogs (expected pCO_2(a) calculated using Eq. 10.9)

Blood gas data for a dog:
pH = 7.146 (7.310–7.420) pCO_2(a) = 36 mmHg (35–45) [HCO_3^-] = 12 mmol/L (17–24)

Example	[HCO_3^-] in patient (mmol/L)	[HCO_3^-] in health (mmol/L)	Compensation factor (mmHg/mmol/L)	pCO_2(a) in health (mmHg)	Expected pCO_2(a) (mmHg)	Difference between measured and expected pCO_2(a) (mmHg)
A	12	21	0.7	40	34	2
B	12	21	1.1	40	30	6
C	12	24	1.1	35	22	14

Example A: Expected pCO_2(a) was calculated using pCO_2(a) and [HCO_3^-] values at the midpoint of their RIs and a compensation factor reflecting the midpoint of expected changes for healthy dogs (Table 10.7). The difference between measured and expected pCO_2(a) values is 2 mmHg and thus within the ±3 guideline. Therefore, the measured pCO_2(a) of 36 mmHg represents an expected respiratory compensation (i.e., compensatory respiratory alkalosis) for the metabolic acidosis.
Example B: Expected pCO_2(a) was calculated using pCO_2(a) and [HCO_3^-] values at the midpoint of their RIs and a compensation factor (1.1) reflecting the highest expected change for healthy dogs (Table 10.7). The difference between measured and expected pCO_2(a) values is 6 mmHg and thus greater than the ±3 guideline. This value suggests that there has not been an appropriate respiratory compensation for the metabolic acidosis, and thus there may be a concurrent respiratory acidosis. However, the 6 mmHg was calculated using values found in experimental healthy dogs rather than ill dogs, and thus the compensation might be appropriate.
Example C: Expected pCO_2(a) was calculated using the lower reference limit for pCO_2(a) (35 mmHg), the upper reference limit for [HCO_3^-] (24 mmol/L), and the higher compensation factor (1.1) for healthy dogs (Table 10.7). The difference between measured and expected pCO_2(a) values is 14 mmHg and thus much greater than the ±3 guideline. This value suggests that there has not been an appropriate respiratory compensation for the metabolic acidosis, and thus there may be a concurrent respiratory acidosis. However, the 14 mmHg was calculated using values found in experimental healthy dogs, and thus the compensation might actually be appropriate.

6. A formula for calculating an expected $pCO_2(a)$ is provided in Eq. 10.9. The expected $pCO_2(a)$ value is frequently calculated using the midpoints of RIs for $pCO_2(a)$ and $[HCO_3^-]$, and a compensation factor reflecting the midpoint of expected changes.

$$\begin{pmatrix}\text{Expected}\\ pCO_2(a)\end{pmatrix} = \begin{pmatrix}pCO_2(a)\\ \text{in healthy dog}\end{pmatrix} + \left(\begin{pmatrix}[HCO_3^-]\\ \text{in patient}\end{pmatrix} - \begin{pmatrix}[HCO_3^-]\\ \text{in healthy dog}\end{pmatrix}\right) \times \begin{pmatrix}\text{compensation}\\ \text{factor}\end{pmatrix} \quad (\mathbf{10.9})$$

Example:
Patient results: pH = 7.146; $pCO_2(a)$ = 36 mmHg; $[HCO_3$ = 12 mmol/L
Central values for a healthy dog (Table 10.8): $pCO_2(a)$ = 40 mmHg; $[HCO_3^-]$ = 21 mmol/L

$$\begin{pmatrix}\text{Expected}\\ pCO_2(a)\end{pmatrix} = (40\text{ mmHg}) + (12\text{ mmol/L} - 21\text{ mmol/L}) \times (0.7\text{ mmHg per mmol/L change})$$
$$= 34\text{ mmHg}$$

F. Guidelines for calculated expected compensations[63]
   1. If a dog has a primary respiratory acid-base disorder and the calculated expected $[HCO_3^-]$ is > 3 mmol/L from the dog's arterial $[HCO_3^-]$, then the compensation is not appropriate and there is a mixed acid-base disorder.
   2. If a dog has a primary metabolic acid-base disorder and the calculated expected $pCO_2(a)$ is > 3 mmHg from the dog's $pCO_2(a)$, then the compensation is not appropriate and there is a mixed acid-base disorder.

G. Examples of mixed acid-base disorders[63]
   1. Respiratory acidosis and metabolic alkalosis: ↑ $pCO_2(a)$, ↑ $[HCO_3^-]$, pH WRI or near RI
      a. Chronic pulmonary disease may reduce ventilation while furosemide or thiazide diuretics cause metabolic alkalosis (see Chapter 9).
      b. Gastric dilation-volvulus may cause loss of gastric HCl and diaphragm compression inhibiting ventilation.
   2. Respiratory alkalosis and metabolic acidosis: ↓ $pCO_2(a)$, ↓ $[HCO_3^-]$, pH WRI or near RI
      a. Pulmonary edema causing hyperventilation and associated with heart failure may cause lactic acidosis due to poor tissue perfusion.
      b. Early septic shock may cause hyperventilation and later cause lactic acidosis due to poor tissue perfusion.
   3. Metabolic acidosis and metabolic alkalosis: depending on the severity of the disorders, the $[HCO_3^-]$ may be ↓, WRI, or ↑
      a. Dogs with chronic renal failure or ketoacidosis have a metabolic acidosis promoting a decrease in $[HCO_3^-]$, and they might have persistent vomiting that promotes increased HCO_3^- production and thus a concurrent metabolic alkalosis.
      b. Dogs with persistent vomiting may develop metabolic alkalosis, promoting an increase in $[HCO_3^-]$, and possibly a metabolic acidosis because of severe dehydration, which may cause poor tissue perfusion and a lactic acidosis that promotes a decrease in $[HCO_3^-]$.
   4. Respiratory acidosis and metabolic acidosis: ↑ $pCO_2(a)$, ↓ $[HCO_3^-]$, ↓ pH
      a. Gastric dilation-volvulus may be present with lactic acidosis (poor perfusion of tissues) and diaphragmatic compression inhibiting ventilation.

b. Pulmonary edema secondary to heart failure may limit ventilation (thus increasing $pCO_2(a)$), and concurrent decreased cardiac output can cause poor tissue perfusion and lactic acidosis.

5. Respiratory alkalosis and metabolic alkalosis: ↓ $pCO_2(a)$, ↑ $[HCO_3^-]$, ↑ pH
   a. Dogs with pulmonary edema due to congestive heart failure may be hyperventilating, causing ↓ $pCO_2(a)$, and be given diuretics that cause a metabolic alkalosis (↑ $[HCO_3^-]$).
   b. Gastric dilation-volvulus may cause loss of gastric HCl and ↑ HCO_3^- production; concurrent pain may cause hyperventilation and loss of $pCO_2(a)$.

HYPOXEMIA AND HYPOXIA

I. Expected $pO_2(a)$
   A. Arterial blood is the only acceptable sample for accurate assessment of oxygenation of blood by the respiratory system. The expected $pO_2(a)$ varies with the $FO_2(I)$, the O_2 content of the inhaled gas, as shown here by paired $FO_2(I)$ in decimal fraction and $pO_2(a)$ in mmHg: 0.2, 95–100; 0.3, 150; 0.4, 200; 0.5, 250; 0.8, 400; and 1.0, 500.[43]
   B. For room air, the $FO_2(I)$ is near 0.2, and thus a $pO_2(a)$ of 95–100 mmHg is expected, although lower pressures are expected at higher altitudes. Some authors consider a $pO_2(a)$ of < 80 mmHg to be hypoxemia,[43] although one could also consider hypoxemia to be present whenever $pO_2(a)$ falls below a valid lower reference limit. Hypoxemic stimulus of chemoreceptors is reported to occur when $pO_2(a)$ is < 70 mmHg[64] or < 60 mmHg.[65]
   C. With the increased use of point-of-care instruments that have O_2 electrodes, $pO_2(v)$ is being measured more often. In free-flowing venous blood in an animal with a $pO_2(a)$ near 95 mmHg, the $pO_2(v)$ is near 40–60 mmHg.
      1. If the sample is collected from an occluded vein, the $pO_2(v)$ will be lower.
      2. It is not appropriate to use a $pO_2(v)$ to assess the oxygenation of blood by the respiratory system.
   D. If collected from capillary blood, the pO_2 should be between the $pO_2(a)$ and $pO_2(v)$ values but close to $pO_2(a)$ if the collection site is "arterialized" (see Analytical Concepts, Section V.C).
   E. Blood gas data in neonates are different from those of adult animals.[49,50]
      1. Compared to adult horses, a foal has lower $pO_2(a)$ (< 40 mmHg) and higher $pCO_2(a)$ values (> 50 mmHg) during its first few hours after birth. The $pO_2(a)$ increases quickly to near adult values by 1 d of age, and the $pCO_2(a)$ slowly changes to adult values by 4–7 d of age.
      2. Similar patterns are seen in neonatal calves. The $pO_2(a)$ approaches adult values by 1 d of age, and the $pCO_2(a)$ approaches adult values by 2 d of age.
      3. Plasma $[HCO_3^-]$ values are also greater initially, corresponding to the greater $pCO_2(a)$.

II. *Hypoxemia*, a decreased $pO_2(a)$, i.e., decreased dissolved O_2 in blood, may cause *hypoxia*, which is decreased O_2 delivery to tissue or decreased utilization of O_2 by tissues. However, there are other causes of hypoxia not due to hypoxemia. Hypoxia has been classified in several ways, including into the following seven groups noted below.[66] Hypoxemic and hypoxic disorders or conditions and the expected laboratory data for hypoxia disorders are listed in Tables 10.9 and 10.10.

Table 10.9. Disorders and conditions that cause hypoxemia[a]
Decreased inhaled O_2 content: high altitude or closed ventilation area
Inhibition or dysfunction of the medullary respiratory center (Table 10.4)
Inhibition or dysfunction of the respiratory muscles (Table 10.4)
Upper airway dysfunction: foreign body, vomitus, or mechanical hypoventilation
Impaired gas exchange at pulmonary capillaries* (Table 10.4)

* A relatively common disorder, condition, or mechanism

[a] Disorders that cause respiratory acidosis with increased pCO_2(a) because of reduced gas exchange in the lungs (Table 10.4) will likely also result in decreased pO_2(a), but the decrease may not be sufficient to be classified a hypoxemia. Changes in pCO_2(v) may reflect pCO_2(a) but less reliably, especially with circulatory failure or during mechanical ventilation. General anesthesia, independent of underlying disorders, may cause or contribute to hypoxemia through multiple mechanisms, including machine or machine-operation problems, hypoventilation if breathing room air, endobronchial intubation, aspiration of gastric contents, compression atelectasis with shunting, and ventilation–perfusion mismatches.

Note: Neonatal RIs should be used for neonates (see text).

Table 10.10. Blood gas results expected for each general type of hypoxia

	Atmospheric	Tidal	Alveolar	Hemoglobic	Stagnant	Histotoxic	Demand
pO_2(a)	↓	↓	↓	WRI	WRI	WRI	WRI
sO_2(a)	↓	↓	↓	WRI	WRI	WRI	WRI
spO_2	↓	↓	↓	?[a]	WRI	WRI	WRI
p_{50}	WRI	WRI	WRI	WRI	WRI	WRI	WRI
O_2ct	↓	↓	↓	↓	WRI	WRI	WRI
pO_2(A)	↓	↓	↓	WRI	WRI	WRI	WRI
a/A	WRI	WRI	↓	WRI	WRI	WRI	WRI
DO_2(Aa)	WRI	WRI	↑	WRI	WRI	WRI	WRI
pO_2(a)/FI	WRI	WRI	↓	WRI	WRI	WRI	WRI
pCO_2(a)[b]	WRI–↓[c]	↑[d]	↑[d]	WRI	WRI	WRI	WRI
pH	WRI–↑[c]	WRI–↓[d]	WRI–↓[d]	WRI	WRI	WRI	WRI

[a] If the hemoglobic (hypemic) hypoxia is caused by severe anemia (Hct < 10 %), the spO_2 may be unreliable. If the hemoglobic hypoxia is caused by methemoglobinemia or carboxyhemoglobinemia, the spO_2 is unreliable by dual-wave pulse oximetry (falsely increased with carboxyhemoglobinemia, whereas methemoglobinemia either falsely increases or decreases spO_2 toward a plateau of 85 %, depending on if the initial sO_2(a) is below or above 85 %).

[b] Changes in pCO_2(v) may reflect pCO_2(a) but less reliably, especially with circulatory failure or during mechanical ventilation.

[c] If the hypoxemia results in the stimulation of respiration, hyperventilation would result in decreased pCO_2(a) and respiratory alkalosis.

[d] If there is inadequate ventilation, there would be an increased pCO_2(a), possibly an increased pCO_2(v), and respiratory acidosis.

A. *Atmospheric hypoxia*: inhalation of atmosphere that has decreased O_2 content (e.g., high altitude, anesthetic problem, or in an airtight box)
   1. The inhalation of atmosphere that has decreased O_2 content causes a decreased pO_2(A), so less O_2 diffuses to blood, thus lowering pO_2(a), and less O_2 binds to Hgb (thus decreasing sO_2). The combination of ↓ pO_2(a) and ↓ sO_2 results in a decreased amount of O_2 in the blood (↓ O_2ct).
   2. If the hypoxemia stimulates the peripheral chemoreceptors (carotid or aortic bodies), increased respirations result in hyperventilation and thus ↓ pCO_2(a) (respiratory alkalosis) if the CO_2 is actually exhaled (see tidal hypoxia in the next section).

B. *Tidal hypoxia*: decreased O_2 uptake because of decreased tidal volume (e.g., respiratory obstruction, hypoventilation caused by anesthetic agents)
   1. The impaired delivery of air to the alveoli has the same effect on the oxygenation of blood as conditions that cause atmospheric hypoxia: ↓ pO_2(a), ↓ sO_2, and ↓ O_2ct.
   2. The impaired respiratory exchange causes impaired excretion of CO_2. Thus, there may be ↑ pCO_2(a) (respiratory acidosis).

C. *Alveolar hypoxia*: decreased O_2 uptake by blood because of decreased alveolar function (e.g., pneumonia, emphysema, pleural effusion, intrapulmonary exudates, pulmonary edema, or pulmonary fibrosis) that may involve right-to-left shunting or ventilation–perfusion mismatches
   1. The impaired diffusion of O_2 has the same effect on the oxygenation of blood as do conditions that cause atmospheric hypoxia: ↓ pO_2(a), ↓ sO_2, and ↓ O_2ct. However, an increased alveolar–arterial O_2 tension gradient (DO_2(Aa)) and a decreased arterial–alveolar O_2 tension ratio (a/A) identify the defective diffusion of O_2 from alveoli to blood.
   2. The impaired respiratory exchange results in impaired excretion of CO_2. Thus, there may be ↑ pCO_2(a) (respiratory acidosis).
   3. A right-to-left shunt results in ↓ pO_2(a) because venous blood with a pO_2(v) of 45–50 mmHg is mixed with arterial blood with a pO_2(a) of 95–100 mmHg (if breathing air).
   4. Ventilation–perfusion mismatches occurring when blood perfuses nonfunctional alveoli (i.e., blood is available for gas exchange, but the gas exchange does not occur) may cause alveolar hypoxia. The blood perfusing affected tissue is not oxygenated (remains as "venous" blood) and then mixes with oxygenated arterial blood from other pulmonary tissue.

D. *Hemoglobic (hypemic) hypoxia*: decreased O_2 bound to Hgb without anemia (e.g., methemoglobinemia or CO poisoning) or decreased Hgb (i.e., anemia)
   1. For these disorders, there is not a defect in the delivery of O_2 to the blood; thus, pO_2(a) is not decreased because pO_2(a) is a measure of the tension created by dissolved O_2 in the plasma and is not dependent on blood [Hgb].
   2. If an animal is anemic, the O_2ct will be decreased because most of the O_2 in blood is bound to Hgb, but sO_2 will not be decreased. However, if an anemic animal's blood contains metHgb or COHgb, spO_2 is unreliable when determined by a dual wavelength oximeter. The spO_2 also may be unreliable (falsely decreased) if anemia is severe (Hct < 10 %).[16]
   3. With methemoglobinemia, both ferrous (Fe^{2+}) and oxidized ferric (Fe^{3+}) heme molecules are present, even within the same Hgb tetramer (valency hybrids). In this state, the ferric heme cannot bind O_2, and the ferrous heme within tetramers containing ferric heme has greater affinity for O_2, so the Hgb saturation or oxygen dissociation curve (Figure 10.4) shifts to the left. Both processes result in hypoxia.[67]

4. CO has a much greater affinity for Hgb than does O_2, and thus even small amounts of CO interfere with delivery of O_2 to tissues.

E. *Stagnant hypoxia* is decreased delivery of O_2 to tissue because of poor blood circulation (e.g., shock, blood vessel occlusion, erythrocytosis, or hyperviscosity syndrome). Arterial blood gas data will not detect an abnormality.

F. *Histotoxic hypoxia* is defective O_2 use by tissues because of interference with metabolic pathways (e.g., some drugs or alcohol). Arterial blood gas data will not detect an abnormality.

G. *Demand hypoxia* is increased O_2 demand by hyperfunctioning cells (e.g., hyperthyroidism, pyrexia). Arterial blood gas data will not detect an abnormality.

III. Decreased sO_2 and spO_2

A. Decreasing sO_2(a) or spO_2 typically indicate the development of hypoxemia. However, as is evident from the shape of the O_2Hgb dissociation curve (Figure 10.4), decreases are not detectable until pO_2(a) is < 90 mmHg, so pO_2(a) may be < 60 mmHg before decreases are detected. A patient with decreased pO_2(a) but sO_2 and spO_2 WRIs may function well at rest but appear hypoxic with mild activity (reduced oxygen reserve).

B. Because decreases in sO_2 and spO_2 typically occur with decreases in pO_2 (hypoxemia), the same conditions should be considered for decreases in each: atmospheric, tidal, or alveolar hypoxias (Table 10.10). However, sO_2(v) (venous, not arterial) may decrease without hypoxemia if there is increased O_2 consumption by tissue (increased aerobic metabolism) or decreased O_2 delivery to tissue, such as occurs with decreased cardiac output or chronic anemia that has resulted in RBC changes promoting O_2 extraction from Hgb.

C. Oxygen supplementation may mask hypoxemic conditions by increasing FO_2(I) and therefore pO_2(a), sO_2, and spO_2.

D. Erroneous spO_2 values may occur when dyshemoglobinemias are present and assessment is done by using a two-wavelength pulse oximeter (see Analytical Concepts, Section III.B.6). With increasing concentrations of COHgb (as in CO poisoning), the spO_2 may remain at > 90 % even when sO_2 decreases to 30 %. This is because sO_2 is the amount of O_2Hgb expressed as a percentage of the Hgb able to bind O_2 (O_2Hgb plus HHgb), thus excluding COHgb. With methemoglobinemia, spO_2 values may be greater or less than the sO_2 values depending on the degree of methemoglobinemia and the actual sO_2 and pO_2(a) in the patient. In contrast, multiwavelength pulse oximeters are able to detect methemoglobinemia with varying degrees of accuracy and provide spO_2 values that are aligned with sO_2.[68,69]

STRONG-ION DIFFERENCE (SID) AND STEWART'S METHOD OF ACID-BASE ANALYSIS

I. Evaluation of electrolyte relationships led to an alternate method of assessing metabolic (nonrespiratory) acid-base disturbances (Stewart's quantitative analysis of acid-base chemistry) and the calculation of SID_x. A complete description of Stewart's method and SID concepts is beyond the scope of this book, but major aspects and a comparison with traditional assessment of acid-base disorders are presented. For a complete analysis of Stewart's method and SID, additional references should be studied.[70–78] In Stewart's method:

A. Electrolyte disturbances are approached in the context of physiochemistry with consideration of equilibrium equations, conservation of mass, and balance of charge (electroneutrality).

B. Nontraditional rather than traditional definitions of acids and bases are used.

C. All electrolytes in biological fluids are considered to be involved in acid-base balance, and maintenance of electroneutrality is expected (Eq. 10.10a).

$$[Na^+] + [K^+] + [fCa^{2+}] + [fMg^{2+}] + [H^+] + [NH_4^+] = [OH^-] + [Cl^-] + [HCO_3^-] + [CO_3^{2-}] + [Alb^{x-}] + [PO_4^{y-}] + [uSA] \quad \textbf{(10.10a)}$$

where x and y represent the variable charges on albumin and phosphates, respectively, and uSA includes SO_4^{2-}, lactate, acetoacetate, β-hydroxybutyrate, other acidic anions of metabolism, and exogenous anions.

$$SID_{true} = (\text{sum of strong cations}) - (\text{sum of strong anions}) = ([Na^+] + [K^+] + [fCa^{2+}] + [fMg^{2+}]) - ([Cl^-] + [uSA]) \quad \textbf{(10.10b)}$$

$$([Na^+] + [K^+] + [fCa^{2+}] + [fMg^{2+}]) - ([Cl^-] + [uSA]) = ([OH^-] + [HCO_3^-] + [CO_3^{2-}] + [Alb^{x-}] + [PO_4^{y-}] - ([H^+] + [NH_4^+]) \quad \textbf{(10.10c)}$$

With words: strong cations – strong anions = weak anions – weak cations
Given SID_{true} = strong cations – strong anions, it follows that
SID_{true} = weak anions – weak cations

$$SID_{true} = ([OH^-] + [HCO_3^-] + [CO_3^{2-}] + [Alb^{x-}] + [PO_4^{y-}]) - ([H^+] + [NH_4^+]) \quad \textbf{(10.10d)}$$

D. Analytes involved in acid-base balance are considered to be either independent or dependent variables.
   1. The *independent variables* are those analytes that are regulated or changed independently of others: pCO_2(a), SID, and A_{TOT} (see the definitions in the next section). They are considered the determinants of pH.
   2. The *dependent variables* are those analytes that change if and only if one of the independent variables changes. The dependent variables include H^+, OH^-, HCO_3^-, and tCO_2.

E. Changes in $[H^+]$ are predicted from changes in concentrations of strong ions. The actual blood $[H^+]$ (i.e., pH) is not measured. To assess the use of SID concepts to predict a patient's plasma pH, SID formulas and published data for A_{TOT} and dissociation constants of weak acids were used to compare measured blood pH and SID-calculated pH in 10 hospitalized dogs. The authors concluded that their SID formula (likely Eq. 10.11c.) and a resultant calculated pH did not reliably predict the patient's measured pH value.[79]

F. Albumin is included as a contributor to acid-base balance, whereas it is not a formalized part of the traditional approach. However, albumin concentrations are considered when interpreting AGs (i.e., recognizing that lower albumin concentrations lower the calculated AG) and are factored into base excess calculations.

G. Hgb is not included as a contributor to acid-base balance, whereas it is a part of the calculated BE_B value that is common in blood gas data.

II. Differences in definitions between the two systems can lead to confusion.[3]

A. Definitions in the traditional assessment of acid-base disorders by using pH, pCO_2(a), and $[HCO_3^-]$

1. An *acid* is a substance that can give off a H^+ (a proton) at a given pH (e.g., hydrochloric acid is capable of giving off H^+).
2. A *base* is a substance that can bind a H^+ (e.g., HCO_3^- is capable of binding H^+).
3. An *acidosis* is a condition in which acidemia (lower pH and higher $[H^+]$) tends to occur.
4. An *alkalosis* is a condition in which alkalemia (higher pH and lower $[H^+]$) tends to occur.

B. Definitions in the Stewart's method
1. *Strong cations* or *strong anions* are those ions that are completely dissociated in physiologic fluids. (Note that the definition does not consider the ions that are complexed with protein or nonprotein ions. Most serum calcium ions, for example, are complexed with anions and are not free or completely dissociated ions. Also, the dissociative constant for NaCl is about 93 % and thus a portion of plasma Na^+ and Cl^- are not free ions.)
   a. Strong cations (Na^+, K^+, fCa^{2+}, and fMg^{2+}): Strong cations (e.g., Na^+) are considered bases because when added to ECF, and if there is not a balancing shift of a strong ion (e.g., removal of K^+ or addition of Cl^-), H^+ shifts from ECF to ICF to make ECF more alkaline.
   b. Strong anions (Cl^-, sulfate, lactate, AcAc, BHB, and other anionic products of metabolism): Strong anions (e.g., Cl^-) are considered acids because when added to ECF, and if there is not a balancing shift of a strong ion (e.g., addition of Na^+ or removal of L-lactate), H^+ shifts from ICF to ECF to make ECF more acidic.
2. An *acidosis* is a condition in which there is an excess of strong anions ("acids") or a deficit of strong cations ("bases"). In the SID approach, an acidosis is present if SID is decreased.
3. An *alkalosis* is a condition in which there is an excess of strong cations ("bases") or a deficit of strong anions ("acids"). In the SID approach, an alkalosis is present if SID is increased.
4. *Weak electrolytes* are those electrolytes that are in equilibria in physiologic fluids.
   a. Weak cations: NH_4^+, H^+, and some γ globulins
   b. Weak anions: HCO_3^-, CO_3^{2-}, PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, and proteins (mostly albumin)
5. A_{TOT} is the sum of the concentrations of all nonvolatile weak acids, including their anionic conjugate base forms ($[HA] + [A^-]$). In plasma, these occur mostly as various anionic forms (A^-) of PO_4 and proteins (albumin and globulins). Although A_{TOT} is an independent variable, $[A^-]$ varies with pH.
6. SID_{true} is the difference between the sum of strong-ion concentrations, i.e., the difference between the sum of strong cation concentrations and the sum of strong anion concentrations (Eq. 10.10b). By rearranging Eq. 10.10a, it is shown that SID_{true} (i.e., strong cations minus strong anions) also equals the difference between the sum of weak anion concentrations and the sum of weak cation concentrations (Eq. 10.10c). This is an important concept for interpreting calculated SID_x values. Thus, SID_{true} could be calculated if the analyte concentrations in Eq. 10.10b or 10.10d could be measured.

C. Using the same concepts as Stewart,[70] Singer and Hastings[80] defined buffer base, which is identical to Stewart's SID.[3]

III. SID_x equations: Several SID equations have been proposed and are denoted here by arbitrary but descriptive subscript numbers. Other than the SID_{true}, these SID_x equations yield values that are estimates based on assumptions. If the assumptions are not correct, the calculated estimates will not be correct. Furthermore, as for many aspects of clinical laboratory data, valid interpretation of the calculated SID_x values depends on having valid reference intervals for each value. Because there is not complete analytical agreement among laboratory methods, any

calculated SID_x value should be compared only with an appropriately established RI for that SID_x calculation. For electrolyte data, one should consider the result differences between direct and indirect potentiometry related to lipoprotein and protein concentrations (see Chapter 9, Sodium (Na^+) Concentration). For proteins, one should recognize that refractometric total protein concentrations are commonly inaccurate (see Chapter 7, Analytical Principles for Total Protein, Albumin, and Globulins). Use of different analytical methods without appropriate reference intervals for SID_x values can cause misinterpretation and misclassification of acid-base status.[81]

A. SID_{true} (Eq. 10.11a, same as Eq. 10.10b; placed here to aid formula comparisons):[71] This equation is consistent with the SID definition but is not useful because concentrations of many analytes in the formula are not routinely measured in clinical laboratories.

$$\begin{aligned} SID_{true} &= (\text{sum of strong cations}) - (\text{sum of strong anions}) \\ &= \left([Na^+] + [K^+] + [fCa^{2+}] + [fMg^{2+}]\right) - ([Cl^-] + [uSA]) \end{aligned} \quad \textbf{(10.11a)}$$

uSA includes SO_4^{2-}, lactate, acetoacetate, β-hydroxybutyrate, other acidic anions of metabolism, and exogenous anions.

$$SID_3 = ([Na^+] + [K^+]) - [Cl^-] \quad \textbf{(10.11b)}$$

$$SID_4 = ([Na^+] + [K^+]) - ([Cl^-] + [\text{L-lactate}^-]) \quad \textbf{(10.11c)}$$

$$SID_5 = \left([Na^+] + [K^+] + [fCa^{2+}]\right) - ([Cl^-] + [\text{L-lactate}^-]) \quad \textbf{(10.11d)}$$

$$SID_6 = \left([Na^+] + [K^+] + [fCa^{2+}] + [fMg^{2+}]\right) - ([Cl^-] + [\text{L-lactate}^-]) \quad \textbf{(10.11e)}$$

$$SID_{\text{adjusted } Cl^-} = [Na^+]_{\text{mean healthy}} - [Cl^-]_{Na^+\text{-adjusted}} \quad \textbf{(10.11f)}$$

B. SID_3 (Eq. 10.11b):[75] This equation estimates the SID_{true} by using three strong-ion concentrations that are routinely measured in serum or plasma. Note that it does not include the concentrations of fCa^{2+}, fMg^{2+}, or any unidentified strong anions (uSA); thus, changes in those analyte concentrations would cause the estimate of SID_{true} to be inaccurate. The [uSA] increases with lactic acidosis and ketoacidosis.

C. SID_4 (Eq. 10.11c):[75,82] This equation estimates the SID_{true} by using four strong-ion concentrations (the three for SID_3 plus L-lactate). Note that it does not include the concentrations of fCa^{2+}, fMg^{2+}, or uSA other than L-lactate (such as D-lactate, BHB, or AcAc); thus, changes in those analyte concentrations would cause the estimate of SID_{true} to be inaccurate.

D. SID_5 (Eq. 10.11d):[82] This equation estimates the SID_{true} by using five strong ions (the four for SID_4 plus fCa^{2+}). Note that it does not include the concentrations of fMg^{2+} or uSA other than L-lactate (such as D-lactate, BHB, or AcAc); thus, changes in those analyte concentrations would cause the estimate of SID_{true} to be inaccurate.

E. SID_6 (Eq. 10.11e):[82] This equation estimates the SID_{true} by using six strong-ion concentrations (those used for SID_5 plus fMg^{2+}). Note that it does not include the concentrations of uSA other than L-lactate (such as D-lactate. BHB, or AcAc); thus, changes in those analyte concentrations would cause the estimate of SID_{true} to be inaccurate.

F. $SID_{\text{adjusted } [Cl^-]}$ (Eq. 10.11f):[74] This equation estimates the SID_{true} by using a Na^+-adjusted $[Cl^-]$ (see Chapter 9 and Eq. 9.3d) and assumes that changes in the $SID_{\text{adjusted } [Cl^-]}$ are due to altered concentrations of HCO_3^-, albumin, PO_4, or "other strong anions" (see Chapter 9 for justification of Na^+-adjusted $[Cl^-]$).

G. Other SID formulas have been proposed that include some but not all of the strong ions.[71,83] Also, there are not standardized names for SID_x formulas, and thus SID_x information should be interpreted carefully. For example, some formulas contain [fCa^{2+}], [fMg^{2+}], or [L-lactate], but not [AcAc] or [BHB]. RIs for SID_x values and possible causes of abnormal SID_x values vary depending on the analytes that are or are not used in the formulas, and with the methods used to measure analyte concentrations.

IV. Comparison of SID_x formulas

A. The relationship between SID_{true} and SID_3 is shown in Eqs. 10.12a and b. SID_3 is a good approximation of SID_{true} if concentrations of fCa^{2+}, fMg^{2+}, and uSA are small, as is typically the case in healthy mammals. However, when there is a high [uSA] as occurs in lactic acidosis or ketoacidosis, the SID_3 value is greater than the SID_{true}, i.e., the SID_3 would overestimate the SID_{true}. Thus, the increased SID_3 would be classified in the SID system as a metabolic alkalosis when the animal has either a lactic acidosis or ketoacidosis (both metabolic acidoses in the traditional acid-base system).

Given Eqs. 10.11a and 10.11b,

$$SID_{true} = SID_3 + \left(\left[fCa^{2+}\right] + \left[fMg^{2+}\right]\right) - [uSA] \quad (\mathbf{10.12a})$$

$$\text{Thus, } SID_3 = SID_{true} - \left[fCa^{2+}\right] - \left[fMg^{2+}\right] + [uSA] \quad (\mathbf{10.12b})$$

B. The relationship between SID_{true} and SID_x values varies with the analytes that are or are not included in the formulas (Eq. 10.11b–e). It is more difficult to compare $SID_{adjusted\ [Cl^-]}$ with the other SID_x values. However, the $SID_{adjusted\ [Cl^-]}$ would also overestimate the SID_{true} if there were an increased [uSA].

C. The changes in SID values that are caused by changes in weak ion concentrations can be seen by examining Eq. 10.13a–c (Eq. 10.13a is the same as Eq. 10.10d). For clinical interpretations, it is important to recognize that plasma concentrations of OH^-, CO_3^{2-}, and H^+ are < 1 μmol/L and that of [NH_4^+] is < 1 mmol/L. Thus, changes in SID_{true} of several mmol/L are mostly due to changes in HCO_3^-, Alb^{x-}, or PO_4^{y-} concentrations (Eq. 10.13a and 10.13b). Eq. 10.13b also shows that SID_{true} depends on three major factors: [HCO_3^-], [Alb^{x-}], and [PO_4^{y-}].

Repeating Eq. 10.10d here as 10.13a:

$$\begin{aligned} SID_{true} &= \left([OH^-] + [HCO_3^-] + \left[CO_3^{2-}\right] + [Alb^{x-}] + [PO_4^{y-}]\right) - ([H^+] + [NH_4^+]) \\ &= (\quad \text{weak anions} \quad) - (\text{weak cations}) \end{aligned} \quad (\mathbf{10.13a})$$

Because typical plasma concentrations of OH^-, CO_3^{2-}, H^+, and NH_4^+ are relatively small,

$$SID_{true} \cong [HCO_3^-] + [Alb^{x-}] + [PO_4^{y-}] \quad (\mathbf{10.13b})$$

$$\text{Given } [Alb^{x-}] + [PO_4^{y-}] \cong [A^-],\ SID_{true} \cong [HCO_3^-] + [A^-] \quad (\mathbf{10.13c})$$

As abnormal concentrations of albumin or PO_4 cause minor changes in [A^-],

$$SID_{true} \cong [HCO_3^-] \quad (\mathbf{10.13d})$$

1. The following concepts and calculations indicate that most changes in SID_{true} are due to changes in $[HCO_3^-]$, marked hypoalbuminemia being another consideration.
   a. If PO_4 and albumin concentrations do not change, changes in $[HCO_3^-]$ change the SID_{true} (Eq. 10.13d). Therefore, the traditional approach and SID approach to acid-base balances have commonality.
      (1) Metabolic alkalosis = ↑ $[HCO_3^-]$ = ↑ SID_{true}
      (2) Metabolic acidosis = ↓ $[HCO_3^-]$ = ↓ SID_{true}
   b. The $[Alb^{x-}]$ contribution to total anionic charge in a dog is estimated to be 0.469 mmol/g of albumin, which amounts to 1.4 mmol/dL at 3 g/dL (14 mmol/L at 30 g/L).[84] Hyperalbuminemic states are rare and, when present, they are mild; thus, they would cause very minor changes in SID_{true}. Considering significant digits, each decrease in albumin of 1 g/dL (10 g/L) would decrease the SID_{true} by 5 mmol/L. Plasma globulins have minor contributions to anionic charge except with certain paraproteinemias.
   c. Serum PO_4 concentrations in most healthy domestic mammals are usually 3–6 mg/dL. Each change of 1 mg/dL in serum $[PO_4]$ is equal to 0.323 mmol/L, but only about half of the PO_4 (0.16 mmol/L) exists as free anions. With PO_4 having an average valence near 1.8 at the pH of blood, each 1 mg/dL change in $[PO_4]$ would change the ionic charge by 0.3 mmol/L (0.16 × 1.8). Therefore, hypophosphatemic states would have negligible effects on SID_{true}. Even a marked hyperphosphatemia (e.g., change from 5 mg/dL to 15 mg/dL) would increase the SID_{true} by only about 3 mmol/L.
2. Another way to examine Eqs. 10.13a and 10.13b is in terms of the buffering capacity of plasma. The electrolytes in those equations that can buffer H^+ are HCO_3^-, PO_4^{y-}, and Alb^{x-}. Thus, in terms of the weak electrolytes, an increase in SID_{true} indicates an increase in the plasma buffer concentration (i.e., traditional metabolic alkalosis). Conversely, a decrease in SID_{true} indicates a decrease in the plasma buffer concentration (i.e., traditional metabolic acidosis).
3. Eq. 10.14a shows the common relationship of SID_{true} and SID_3. When using SID_3 values to classify acid-base changes, it is important to remember that the SID_3 does not accurately reflect SID_{true} when there are increased concentrations of [uSA] as occurs with lactic acidosis, ketoacidosis, and other potential organic acidoses.

Given Eq. 10.12a and that typical plasma $[fCa^{2+}]$ and $[fMg^{2+}]$ are relatively small when compared to SID_{true},

$$SID_{true} \cong SID_3 - [uSA] \quad (\mathbf{10.14a})$$

Given Eqs. 10.13c and 10.14a,

$$SID_3 - [uSA] \cong [HCO_3^-] + [A^-], \text{ so} \quad (\mathbf{10.14b})$$

$$SID_3 \cong [HCO_3^-] + [A^-] + [uSA]$$

Considering Eq. 10.11b, Eq. 10.14b, and that

$$AG = ([Na^+] + [K^+]) - ([Cl^-] + [HCO_3^-], \text{ then}$$

$$AG = SID_3 - [HCO_3^-], \text{ so} \quad (\mathbf{10.14c})$$

$$AG \cong [A^-] + [uSA]$$

4. As shown in Eq. 10.14b, changes in SID_3 occur when the sum of $[HCO_3^-]$, $[Alb^{x-}]$, $[PO_4^{y-}]$, and [uSA] changes. If $[Alb^{x-}]$, $[PO_4^{y-}]$, and [uSA] stay constant, changes in SID_3 reflect changes in $[HCO_3^-]$; ↑ SID_3 reflects ↑ $[HCO_3^-]$ and thus a metabolic alkalosis, and ↓ SID_3 reflects ↓ $[HCO_3^-]$ and thus a metabolic acidosis. However, the relationship between SID_3 and $[HCO_3^-]$ is less predictable, or is not present, when there are changes in $[Alb^{x-}]$, $[PO_4^{y-}]$, and [uSA].
5. The SID calculated by Eq. 10.13c has been called SID_e, short for $SID_{effective}$, i.e., $SID_e = [HCO_3^-] + [A^-] \approx SID_{true}$, and it is equivalent to buffer base. An estimate of $[A^-]$ can be calculated from serum concentrations of albumin and phosphorus using pH-based formulas or various simplifications of them.[23,84] When SID is calculated from the strong-ion concentrations used in any of Eqs. 10.11, it has been called SID_a, for $SID_{apparent}$ (the apparent SID based on the SID equation used). The difference between SID_a and SID_e (e.g., $SID_3 - SID_e$ or $SID_6 - SID_e$) is the *strong-ion gap (SIG)*, the difference between charges of unmeasured strong anions and unmeasured strong cations, i.e., those strong anions and strong cations not used in the SID calculations.[85] For example, unmeasured ions would always include D-lactate, sulfate, fatty acids, and ketoacids, and could include phosphate, L-lactate, fCa^{2+}, and fMg^{2+} if these ions were not included in simplified formulas. Therefore, SIG is similar to AG (see Chapter 9), but it is not affected by changes in pH or changes in concentrations of albumin, and may not be affected by concentrations of lactate, PO_4, fCa^{2+}, or fMg^{2+} if complete calculations are used. When there is a metabolic acidosis caused by addition of unmeasured strong anions (e.g., with lactic acidosis or ketoacidosis), SID_a increases without an increase in SID_e, and therefore SIG increases (as does AG). Like AG, SIG also decreases with increases in unmeasured cations (e.g., some monoclonal immunoglobulins) or with false increases in $[Cl^-]$ (e.g., bromide treatment). Although SIG calculations include more ions than the AG calculation, making it theoretically better at detecting changes in actual unmeasured anions, it has relatively high imprecision from use of multiple equations with multiple assumptions and additive errors from each measurement.

D. Eq. 10.14c shows the relationship between AG and SID_3. As described in Chapter 9, the AG is mostly due to the ionic charge of proteins (mostly albumin) if there are not increased concentrations of other organic anions. The AG calculation can also be used to calculate other SID_x values, i.e., SID_{AG} (Eq. 10.15).
   1. Because an increased AG typically indicates an increased concentration of anions other than HCO_3^- or Cl^-, the increase in AG estimates the increased [uSA]. This SID_{AG} formula would provide a more accurate estimate of SID_{true} than does the SID_3 formula when there is a metabolic acidosis. However, the need to calculate any SID_x value is minimal if a decreased $[HCO_3^-]$ has already been recognized, and an associated increase in AG already indicates an increased [uSA].
   2. The difference between $AG_{patient}$ and AG_{health} (part of Eq. 10.15) is called ΔAG and is described above in mixed acid-base disorders.

Given Eq. 10.11a and that an increased AG ≅ increased [uSA], where increased $AG = AG_{patient} - AG_{health}$,

$$SID_{AG} = ([Na^+] + [K^+]) - ([Cl^-] + (AG_{patient} - AG_{health})) \quad (\mathbf{10.15})$$

Table 10.11. Classification of acid-base disorders from pCO_2(a), A_{TOT}, and SID

Change in independent variable		Change in measured analyte	Classification	Expected dependent variable change	
				$[HCO_3^-]$	pH
pCO_2(a)	↓	↓ pCO_2(a)[a]	Respiratory alkalosis	WRI	↑
	↑	↑ pCO_2(a)[a]	Respiratory acidosis	WRI	↓
A_{TOT}	↑	↑ Albumin[b]	Hyperalbuminemic acidosis	↓	↓
	↑	↑ PO_4	Hyperphosphatemic acidosis	↓	↓
	↓	↓ Albumin[c]	Hypoalbuminemic alkalosis	↑	↑
SID_t/SID_3[d]	↑/↑	↑ Na^+	Hypernatremic or contraction alkalosis[e]	↑	↑
	↑/↑	↓ Cl^-	Hypochloremic alkalosis	↑	↑
	↓/↓	↓ Na^+	Hyponatremic or dilutional acidosis[f]	↓	↓
	↓/↓	↑ Cl^-	Hyperchloremic acidosis	↓	↓
	↓/WRI	None[g]	Metabolic acidosis and ↑ [uSA][h]	↓	↓
	↓/↑	Variable[i]	Metabolic acidosis and ↑ [uSA][j]	↓	↓

[a] Changes in pCO_2(v) may reflect pCO_2(a) but less reliably, especially with circulatory failure or during mechanical ventilation

[b] If hyperproteinemia, then hyperproteinemic acidosis

[c] If hypoproteinemia, then hypoproteinemic alkalosis

[d] $SID_t = SID_{true}$

[e] With loss of solute-poor water, $[Na^+]$ and $[Cl^-]$ increase proportionately, but $[Na^+]$ increases by a greater increment than $[Cl^-]$. Therefore, SID increases (but mildly).

[f] Opposite explanation of hypernatremic alkalosis

[g] Concentrations of Na^+, K^+, and Cl^- are all WRI.

[h] Because the SID_3 is WRI, the acidosis would be recognized if it is known that either a ↓ $[HCO_3^-]$ or an ↑ AG (which indicates ↑ [uSA]) is present.

[i] Could be found in hyponatremic, normonatremic, or hypernatremic animals. The SID_3 is increased because the difference between $[Na^+ + K^+]$ and $[Cl^-]$ is increased.

[j] The acidosis would be recognized by the presence of either a ↓ $[HCO_3^-]$ or an ↑ AG (which indicates ↑ [uSA] and a condition promoting a ↓ $[HCO_3^-]$).

V. Interpretation of abnormal SID_3 values

A. According to Stewart's method and definitions, changes in SID_{true} create either an alkalosis or an acidosis. A metabolic acidosis can be associated with a decreased SID, an increased A_{TOT}, or both. A metabolic alkalosis can be associated with an increased SID, a decreased A_{TOT}, or both. SID should always be interpreted in conjunction with A_{TOT}. Based on the definitions and the calculation of SID_3, the major types of acid-base disorders are listed in Table 10.11. Note that the expected changes in the dependent variables (i.e., $[HCO_3^-]$ and pH in right columns) are those expected in the traditional approach to acid-base disorders in which ↑ $[HCO_3^-]$ is a metabolic alkalosis and ↓ $[HCO_3^-]$ is a metabolic acidosis.

B. Based on the definitions, changes in concentrations of two ions may cause a mixed acid-base disorder, e.g., loss of plasma H_2O could result in concurrent hypernatremic alkalosis and hyperalbuminemic acidosis. Conversely, rapid infusion of saline, for which SID and A_{TOT} are both zero, could result in concurrent acidosis from a dilutional increase in SID and

alkalosis from a dilutional decrease in A_{TOT}, with the acidosis predominating. The concurrent alkalosis and acidosis in the Stewart method raises the question of whether there is a noSIDosis. ☺

VI. As recommended by some authors, SID_x values should be interpreted with routine blood gas values (pH, arterial or venous pCO_2, and $[HCO_3^-]$) to determine whether the animal has an acid-base problem and whether the problem is respiratory or nonrespiratory (metabolic).[73,74] If the problem is a nonrespiratory acid-base disturbance, then the contributions of nonbicarbonate electrolytes can be explored with Stewart's method.

VII. Prior to the proposed use of the strong-ion theory, an understanding of an animal's acid-base and electrolyte disorder was obtained through the interpretation of pH, pCO_2(a) or pCO_2(v), $[HCO_3^-]$, BE(B), $[Na^+]$, $[K^+]$, $[Cl^-]$, AG, and albumin concentration (see the preceding Acid-Base Abnormalities section and Chapter 9). Evaluation of such information has provided and will continue to provide an understanding of an animal's pathologic state. An extensive review and critique of Stewart and bicarbonate-centered approaches is available.[76] The authors also state that SID (SID_{true} in this chapter) approximates $[HCO_3^-]$ (shown in Eq. 10.13d).

VIII. As those who study SID theory and its application state, the SID approach requires species-specific values for $[A^-]$, i.e., the sum of the charge concentrations of nonvolatile buffers, serum or plasma proteins, and PO_4.[82] These values have been problematic. As stated earlier (in the preceding Section II.B.5) and in Eq. 10.13c, plasma $[A^-]$ consists primarily of the various anionic forms of PO_4 and proteins (albumin and globulins).

A. There have been attempts to calculate plasma A_{TOT} and $[A^-]$ for healthy cattle,[86,87] dogs,[84] cats,[82] and birds.[88] The calculated values (mean ± SD) were obtained by using results from only 10 dogs, 10 cats, 12 pigeons, and 9 calves (or pooled bovine and ovine plasma). For most of the data, the variations in the calculated plasma A_{TOT} values reflect but underestimate the variations in plasma protein and PO_4 concentrations in such samples from healthy animals.

B. Using those calculated mean plasma A_{TOT}-derived and $[A^-]$ values and measured $[HCO_3^-]$, SID_{true} can be calculated (Eq. 10.13c). However, because there are biological variations in the serum or plasma concentrations of PO_4 and proteins, a mean and $[A^-]$ value may not be appropriate for all healthy animals and are definitely not appropriate for animals that have abnormal protein and PO_4 concentrations. Also because of the lack of agreement in many clinical assays, plasma and $[A^-]$ values calculated for one set of assays may not be appropriate for another set of assays.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 11

CALCIUM, PHOSPHORUS, MAGNESIUM, AND THEIR REGULATORY HORMONES

Michael A. Scott and Steven L. Stockham

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Table 11.1. Abbreviations and symbols in this chapter	
[x]	x concentration (x = analyte)
$1,25(OH)_2D$	1,25-dihydroxyvitamin D; $1,25(OH)_2D2$ or $1,25(OH)_2D3$
$1,25(OH)_2D2$	1,25-dihydroxyvitamin D2 (1,25-dihydroxyergocalciferol, ercalcitriol)
$1,25(OH)_2D3$	1,25-dihydroxyvitamin D3 (1,25-dihydroxycholecalciferol, calcitriol)
25OHD	25-hydroxyvitamin D; 25OHD2 or 25OHD3
25OHD2	25-hydroxyvitamin D2 (25-hydroxyergocalciferol, ercalcidiol)
25OHD3	25-hydroxyvitamin D3 (25-hydroxycholecalciferol, calcidiol)
Ca^{2+}	Calcium
CaSR	Calcium-sensing receptor
ECF	Extracellular fluid
EDTA	Ethylenediaminetetraacetic acid
fCa^{2+}	Free ionized calcium
FGF23	Fibroblast growth factor 23
fMg^{2+}	Free ionized magnesium
GFR	Glomerular filtration rate
GH	Growth hormone
HHM	Humoral hypercalcemia of malignancy
ICF	Intracellular fluid
IL-x	Interleukin x (x for Arabic number of specific interleukins)
Mg^{2+}	Magnesium
NPA	Nonprotein anions
Pi	Inorganic phosphorus
PO_4	Phosphate, including PO_4^{3-}, HPO_4^{2-}, and $H_2PO_4^-$
PTH	Parathyroid hormone
[PTH]	Immunoreactive parathyroid hormone concentration
PTHrP	Parathyroid hormone–related protein
RBC	Red blood cell (erythrocyte)
RI	Reference interval
RIA	Radioimmunoassay
SI	Système international d'unités (International System of Units)
tCa^{2+}	Total calcium
tMg^{2+}	Total magnesium
TNF	Tumor necrosis factor
TRPM6	Transient receptor potential melastatin subtype 6
URL	Upper reference limit
VDBP	Vitamin D–binding protein
WRI	Within reference interval

TOTAL AND FREE CALCIUM (tCa^{2+}, fCa^{2+}) CONCENTRATIONS

I. Physiologic processes

A. Serum or plasma Ca^{2+} is distributed into three major fractions. All Ca^{2+} in body fluids is ionized, but some is free, and some is reversibly bound to anionic molecules because of charge interactions. Similar percentages for the fractions are reported for people,[1] dogs,[2] and horses.[3,4]

1. General concepts
   a. fCa^{2+}: About 50 % of [tCa^{2+}] is *fCa^{2+}*, which is present as free ions in plasma H_2O. The [fCa^{2+}] reflects the biologically active portion of [tCa^{2+}] that is hormonally regulated and contributes to pathologic states. In clinical jargon, fCa^{2+} is usually called *ionized calcium* and is abbreviated as iCa, iCa^{2+}, or just Ca^{2+}. Referring to the unbound Ca^{2+} as fCa^{2+}, as is done in this book and by other clinical chemists,[5] avoids incorrectly implying that Ca^{2+} bound to anions is not ionized, and is analogous to using tT_4 and fT_4 for total and free thyroxine, respectively. It is also helpful for understanding the effects of pH on [fCa^{2+}].
   b. Anion-bound Ca^{2+}
      (1) Bound to anionic proteins: About 40–45 % of tCa^{2+} is bound to negatively charged sites on proteins (80 % to albumin and 20 % to globulins).[1] Since binding is charge dependent, changes in blood pH slightly alter Ca^{2+} binding and thus slightly alter distribution of Ca^{2+} between bound and free fractions. Human albumin has potentially 30 Ca^{2+}-binding sites, but only 4 appear to be important at physiologic pH.[6]
      (2) Bound to NPA: About 5–10 % of tCa^{2+} is bound by charge to citrates, PO_4, lactate, and other small, diffusible anions.
      (3) Often-used terms might suggest that there are different types of Ca^{2+} interactions with anionic proteins and with NPA because the former is often referred to as "protein-bound Ca^{2+}" and the latter as "anion-bound Ca^{2+}" or "complexed Ca^{2+}." However, both fractions involve anions and reversible charge-based complexes.
2. The amount of Ca^{2+} in each plasma fraction is dependent on several factors.
   a. The [fCa^{2+}] is regulated by hormones, primarily PTH and vitamin D.
   b. The [protein-bound Ca^{2+}] is mostly dependent on [albumin] but also is affected by [anionic globulins].
   c. The concentration of Ca^{2+} complexed to NPA is primarily dependent on the concentrations of those anions.
   d. Changes in pH affect the binding of Ca^{2+} to proteins and NPA.
   e. Other less characterized factors affecting the proportion of free and bound Ca^{2+} include [Na^+], [Cl^-], and [fatty acids].[7]

B. Major factors that determine serum [tCa^{2+}] (Figure 11.1)
1. Age
   a. Young dogs (6–24 wk old) have serum [tCa^{2+}] ~1–2 mg/dL greater than mature dogs.[8,9] Greater [fCa^{2+}] in puppies (4 d to 12 wk) contributes to the greater [tCa^{2+}], but typically by < 1 mg/dL.[10]
   b. Some kittens up to at least 8 wk of age had a [tCa^{2+}] up to as much as 3 mg/dL greater than values expected in adults.[11]
   c. The [tCa^{2+}] in beef calves from birth to 72 h was as much as 2.5 g/dL and usually 0.5–2.0 g/dL greater than the URL for adult cattle;[12] age-related data were not found for older calves.
   d. Alpaca crias ≤ 6 mo of age have a mean [tCa^{2+}] about 10 % greater than alpacas > 6 mo of age.[13]
   e. As a group, young foals (1–60 d) have tCa^{2+} concentrations similar to those of adults,[14] but concentrations of tCa^{2+} and fCa^{2+} were lower in 1- to 7-d-old foals than in adult horses.[15]
2. Because serum Ca^{2+} concentrations in common chemistry profiles represent [tCa^{2+}], a decrease in serum protein concentration (especially hypoalbuminemia) causes a decrease in the protein-bound Ca^{2+} and thus may cause hypocalcemia. A decreased concentration of protein-bound Ca^{2+} does not alter the hormonal regulation of [fCa^{2+}].

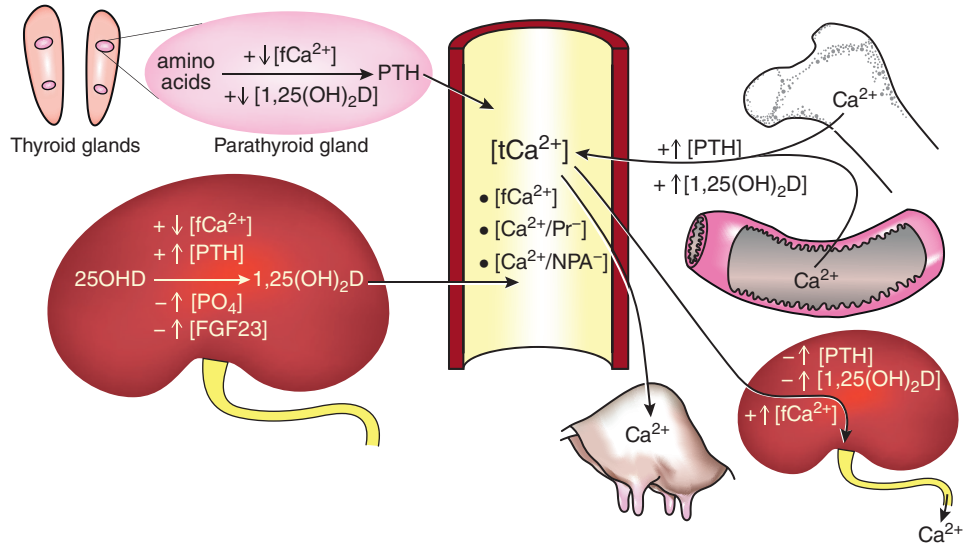


Figure 11.1. Relationships of calcium kinetics and the production of PTH and $1,25(OH)_2D$. Note: Horse kidneys lack 1α-hydroxylase and thus do not form $1,25(OH)_2D$ as shown here.

- PTH production in parathyroid glands is stimulated by ↓ $[fCa^{2+}]$ and ↓ $[1,25(OH)_2D]$; it is inhibited by ↑ $[fCa^{2+}]$ and ↑ $[1,25(OH)_2D]$.
- Conversion of 25OHD to $1,25(OH)_2D$ in kidneys is catalyzed by 1α-hydroxylase. The activity of 1α-hydroxylase is promoted by ↓ $[fCa^{2+}]$ and ↑ [PTH]; it is inhibited by ↑ $[PO_4]$ and ↑ [FGF23].
- Ca^{2+} mobilization from bone and Ca^{2+} absorption in intestine are promoted by ↑ $[1,25(OH)_2D]$ and ↑ [PTH]. Less Ca^{2+} mobilization and absorption occur if there is ↓ $[1,25(OH)_2D]$ or ↓ [PTH].
- Urinary excretion of Ca^{2+} is enhanced by ↑ $[fCa^{2+}]$; excretion is reduced by ↓ $[fCa^{2+}]$, ↑ [PTH], and ↑ $[1,25(OH)_2D]$. Increased excretion of anions may obligate Ca^{2+} excretion and thus decrease $[fCa^{2+}]$.
- During lactation, a large amount of Ca^{2+} is excreted via milk.
- Ca^{2+} is present in plasma in three forms: fCa^{2+}, Ca^{2+} bound to proteins, and Ca^{2+} bound to small NPAs such as citrate and PO_4.

+, positive effector (stimulates the process); –, negative effector (inhibits the process); Ca^{2+}/Pr^-, calcium bound to protein; and Ca^{2+}/NPA^-, calcium bound to NPAs

3. Absorption of Ca^{2+} in intestine (mostly in the ileum, but from duodenum to colon)
   a. In dogs, cats, and cattle, intestinal Ca^{2+} absorption occurs paracellularly through epithelial tight junctions, and transcellularly, aided by intracellular calbindin (a Ca^{2+}-binding protein). Vitamin D facilitates both paths by making tight junctions more permeable to Ca^{2+} and by inducing mucosal epithelial cells to synthesize calbindin. PTH activity augments vitamin D actions to increase Ca^{2+} absorption, mostly by stimulating $1,25(OH)_2D$ production.
   b. Based on renal failure studies in horses, Ca^{2+} absorption in the equine intestine depends less on vitamin D and more on the amount of dietary Ca^{2+}.[16] Equine kidneys lack 1α-hydroxylase, but horses do have low plasma concentrations of $1,25(OH)_2D$ along with a predominance of 25OHD2, small quantities of 24,25-dihydroxychlecalciferol ($24,25(OH)_2D3$), and very little or no 25OHD3 (see Vitamin D Concentration section for vitamin D terms).[17,18]

4. Ca^{2+} resorption from or deposition in bone
   a. PTH stimulates bone remodeling by binding to its receptor on osteoblasts, osteocytes, and osteoblast precursor cells. The major bone resorptive effects of PTH occur indirectly by osteoclasts in response to a protein produced by stimulated osteocytes and osteoblasts. This protein (receptor activator of nuclear factor-kappa B ligand, RANKL) binds to its osteoclast receptor (RANK) and stimulates osteoclasts. Stimulated osteoclasts adhere to the bone surface, forming sealed bone-apposing spaces into which they secrete lytic enzymes and H^+; they remove the degraded inorganic mineral and organic collagen matrix from the resorption pit by endocytosis, and the resorbed Ca^{2+} and other substances are released into the ECF.[19,20]
   b. Vitamin D enhances Ca^{2+} resorption from bone by promoting osteoclast activity and osteoclastogenesis through binding to its receptor on osteoclasts, osteoblasts, and osteocytes. Stimulated osteoblasts and osteocytes promote osteoclast-mediated resorption through the generation of RANKL.[21]
   c. Calcitonin blocks osteoclastic osteolysis through direct changes in osteoclasts and by inhibiting osteoclastogenesis.[22] However, continuous PTH stimulation can cause osteoclasts to escape from the suppressive effects of calcitonin.
5. Resorption of fCa^{2+} from tubular fluid in renal tubules
   a. Ca^{2+} (free and bound to NPA) passes freely through the glomerular filtration barrier, but most protein-bound Ca^{2+} should not pass through. About 65 % of filtered Ca^{2+} is resorbed in proximal tubules, mostly passively down a concentration gradient formed by Na^+ and H_2O resorption. A smaller amount of Ca^{2+} is resorbed actively in response to PTH. About 25 % of filtered Ca^{2+} is resorbed in the thick ascending limb of the loop of Henle, mostly passively down an electrochemical gradient established by a Na^+-K^+-$2Cl^-$ cotransporter and a recycling of K^+,[23] but also by changes in tight junctions secondary to PTH stimulation of CaSRs. Fine regulation of Ca^{2+} resorption occurs transcellularly in the distal nephron with the aid of specific apical transporters, intracellular calbindin, and a basolateral Na^+-Ca^{2+} exchanger. Vitamin D and PTH promote Ca^{2+} resorption in the distal nephron by upregulating calbindin and apical transporters.
   b. Angiotensin II stimulates the resorption of Na^+ and Ca^{2+} in the proximal and distal tubules.

C. Ca^{2+} and PO_4 interaction
1. Metastatic calcification: A long-standing concept is that metastatic mineralization of tissues (especially in kidneys and lungs) tends to occur when the product of the plasma [tCa^{2+}] times the plasma [Pi] (both in mg/dL) exceeds 70. This dogma is based on the chemical laws of mass action for the formation of a $Ca_3(PO_4)_2$ precipitate; more $Ca_3(PO_4)_2$ forms as the [Ca^{2+}] or [PO_4^{3-}] in a solution increases. However, this concept does not apply to physiologic and pathologic conditions.
   a. In tissues other than blood
      (1) There are inhibitors of tissue calcification. These include fetuin-A, matrix Gla-protein, osteoprotegerin, osteopontin, osteocalcin, and pyrophosphate.[24,25]
      (2) When tissue calcification occurs with hypercalcemia or hyperphosphatemia, multiple active cellular processes are involved, including accumulation of Ca^{2+} or PO_4 in cells and osteogenic differentiation of cells.
   b. In plasma
      (1) In health, only fCa^{2+} and free PO_4^{3-} are available for formation of $Ca_3(PO_4)_2$, whereas the "mass law" concept is applied to measured total concentrations; free ion concentrations would be needed to predict increases in the formation of $Ca_3(PO_4)_2$.

(2) When Ca^{2+} and PO_4^{3-} were added to human plasma, precipitation did not occur until the $Ca^{2+} \times PO_4$ product exceeded 200.[26]
(3) When the plasma solubility of calcium phosphate is exceeded in vivo, colloidal particles form and are rapidly removed by macrophages.[27]
(4) If a $[tCa^{2+}] \times [Pi]$ product > 70 were truly indicative of mineralization, many young healthy animals, which have greater plasma $[tCa^{2+}]$ and especially [Pi], would have mineralization problems, but they do not.

II. Analytical concepts of $[tCa^{2+}]$
A. Sample
1. Serum is the preferred sample for $[tCa^{2+}]$. Heparinized plasma also may be used. Serum $[tCa^{2+}]$ is stable for at least 3 d at 4 °C and 23 °C;[28] another study found that serum $[tCa^{2+}]$ is stable for at least 7 d at 9 °C or room temperature.[29] The $[tCa^{2+}]$ remained stable for 14 d in plasma and serum harvested from bovine whole blood stored at 4 °C, and 12-mo stability was shown for serum and heparinized plasma stored at –80 °C.[30]
2. Blood anticoagulants that substantially bind Ca^{2+} (EDTA, citrate, and oxalate) should not be used in samples for Ca^{2+} assays.
3. In people, prolonged venous occlusion during blood collection may increase the $[tCa^{2+}]$ by 0.5–1.0 mg/dL.[1]
B. Common clinical assays are photometric assays that measure $[tCa^{2+}]$ (free + bound).
1. o-Cresolphthalein assay: o-Cresolphthalein reacts with Ca^{2+} to form a red complex.
2. Arsenazo III dye colorimetric assay: Bound Ca^{2+} is liberated from anions and then Ca^{2+} reacts with Arsenazo III dye to produce a colored complex.
C. Unit conversion: mg/dL × 0.2495 = mmol/L, and mEq/L × 0.5 = mmol/L (SI unit)[31]

III. Analytical concepts of $[fCa^{2+}]$
A. Sample
1. Acceptable samples include whole blood (for some point-of-care analyzers), serum, heparinized whole blood, and heparinized plasma; anticoagulants that bind more Ca^{2+} (EDTA, citrate, and oxalate) cannot be used in samples for fCa^{2+} or tCa^{2+} assays. Because of the potential changes in $[fCa^{2+}]$ caused by preanalytical variations, it is essential that patient samples and samples for determination of RIs be collected and processed by using uniform and stringent criteria.[32] The gel in silicone-separator tubes may cause mild (~0.08 mg/dL) false increases in $[fCa^{2+}]$.[33]
2. Whether $[fCa^{2+}]$ is measured in blood collected into a syringe or a tube, an excess amount of heparin should be avoided because heparin (a polyanion) binds Ca^{2+}. The $[fCa^{2+}]$ may decrease 5 % to 50 % depending on the amount of sodium heparinate (commonly called heparin) in the sample.[34]
a. When lithium or sodium heparin is used, the heparin concentration should be 15 IU/mL blood or less.[35] Some heparin tubes are designed to produce that concentration, e.g., a 4 mL tube containing 60 IU of lithium heparin. Collecting less blood than the designed volume results in an excess heparin concentration and a falsely low $[fCa^{2+}]$.
(1) Collecting 1.0 mL blood into a 4 mL tube produces a heparin concentration of 60 IU/mL. That concentration has been shown to lower $[fCa^{2+}]$ from 1.25 mmol/L to near 1.15 mmol/L (from 5.0 mg/dL to 4.6 mg/dL).[36]

(2) Other studies indicate the following heparin-induced negative biases in [fCa^{2+}]: 0.03 mmol/L @ 15 IU/mL, 0.15 mmol/L @ 50 IU/L, and 0.19 mmol/L @ 100 IU/L (~0.1, 0.6, and 0.8 mg/dL, respectively).[37]

b. Two factors can falsely decrease [fCa^{2+}] when collecting blood into a syringe containing liquid heparin: (i) dilution and (ii) binding to heparin. Assuming the heparin solution has a concentration of 1000 IU/mL and the dead space of the syringe has a volume near 0.1 mL:
   (1) Drawing to a total volume of 2 mL, the blood is diluted by 5 % and the heparin concentration is near 50 IU/L. The 5 % dilution alone would lower the [fCa^{2+}] from 1.2 mmol/L to 1.14 mmol/L. The excess heparin binding alone would lower the [fCa^{2+}] to near 1.05 mmol/L (i.e., an original 4.8 mg/dL to 4.2 mg/dL).
   (2) Drawing to a total volume of 1 mL, the blood is diluted by 10 % and the heparin concentration is near 100 IU/L. The dilution alone would lower the [fCa^{2+}] from 1.2 mmol/L to 1.08 mmol/L. The excess heparin binding alone would lower the [fCa^{2+}] to near 0.93 mmol/L (i.e., original 4.8 mg/dL to 3.7 mg/dL).

c. In an experiment, canine blood was collected into syringes (to the 1 mL mark) that contained varying amounts of heparin. Heparin had been drawn into the syringes and then expelled to leave various residual amounts. The [fCa^{2+}] in samples collected after heparin was forcibly expelled three times was considered the most accurate value. The [fCa^{2+}] decreased by 0.2 mmol/L (0.8 mg/dL) with about 0.1 mL of excess heparin (the amount in the syringe dead space and needle), decreased 0.5 mmol/L (2 mg/dL) with about 0.2 mL of excess heparin (heparin in the dead space plus 0.1 mL in the syringe), and decreased 0.7 mmol/L (2.8 mg/dL) with about 0.35 mL of excess heparin (heparin in the dead space plus 0.25 mL in the syringe).[38]

3. To minimize preanalytical error when measuring [fCa^{2+}] with a blood gas analyzer, the use of special calcium-titrated heparin syringes is recommended. The Ca^{2+} content in the calcium-titrated heparin approximates the expected midpoint concentration of fCa^{2+} in human plasma and should reduce the binding of patient fCa^{2+} to heparin. Because the measured [fCa^{2+}] represents Ca^{2+} from the calcium-titrated heparin and Ca^{2+} in the patient's plasma, RIs based on similarly collected samples would be most appropriate.
4. Because changes in [fCa^{2+}] may be affected by dietary intake and postprandial alkalemia, overnight fasting of dogs before sample collection has been recommended.[39] Similar changes are expected in cats and horses.
5. Predictions of [fCa^{2+}] from [tCa^{2+}] based on protein concentrations or complex multivariate models are just that: predictions. They may aid recognition of clinically significant hyper- and hypocalcemia compared to assessment of [tCa^{2+}] alone, but they can be misleading; formulas are not universally applicable because of substantial differences in analyzers, assay methods, and patient populations. Recognizing free hypocalcemia or free hypercalcemia requires appropriate measurement of [fCa^{2+}] and appropriate RIs.

B. Sample processing
1. To measure the actual [fCa^{2+}] in the patient, samples should be processed anaerobically and promptly to avoid the effect of pH changes on [fCa^{2+}]: raising the pH decreases [fCa^{2+}], whereas lowering the pH increases [fCa^{2+}].[1] The [fCa^{2+}] changes by about 5 % for each 0.1 in vitro change in pH (caused by CO_2 escape). Exposing sera to air by simply inverting tubes containing sera and air lowered the [fCa^{2+}] by a mean of 0.1 mmol/L and raised the pH values by a mean of 0.35.[40]

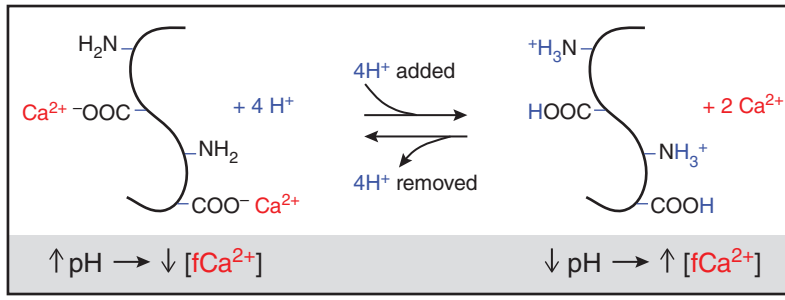


Figure 11.2. Conceptual illustration of the effects of a changing plasma or serum pH on [fCa^{2+}]. Schematic S-shaped protein molecules are shown with amine (NH_2) and carboxyl (COOH) groups. When the medium becomes more acidic (H^+ added), H^+ binds to the protein, thus displacing Ca^{2+} and increasing the [fCa^{2+}]. When the medium becomes more alkaline (H^+ removed), more Ca^{2+} becomes bound to the protein molecules and the [fCa^{2+}] decreases. A similar but relatively minor shifting of bound and free Ca^{2+} occurs with NPAs in serum or plasma. Similar effects occur with [fMg^{2+}].

a. Albumin and other protein molecules have negatively and positively charged components that bind cations (e.g., Ca^{2+} and H^+) and anions (e.g., Cl^-), respectively. The binding of some ions can displace others or alter other binding sites. Plasma or serum pH affects available binding sites and alters the binding of Ca^{2+} to proteins. This relationship is illustrated in Figure 11.2.
b. A greater pH increases the available negative-charge sites on albumin and other proteins because fewer H^+ ions are present to bind them. This leads to increased protein-bound Ca^{2+} and thus decreased [fCa^{2+}]. Aerobic sample handling leads to escape of CO_2 to air, an increase in pH, and thus a falsely low [fCa^{2+}].
c. A lower pH induces the opposite changes and thus increases [fCa^{2+}]. Delayed analysis of whole blood allows blood cell metabolic pathways to produce H^+, causing a falsely increased [fCa^{2+}].
d. The [fCa^{2+}] normalized to a pH of 7.4 (see following Section C.3) should be unchanged with only mild to moderate shifts in pH.

2. If not testing whole blood, serum or plasma should be harvested within 1 h of blood collection and with minimal exposure to air to reduce potential errors created by the loss of CO_2 and the generation of lactate and H^+ by blood cells.
3. Sample storage: In anaerobically collected and stored heparinized whole blood, [fCa^{2+}] was reportedly stable for 9 h at 4 °C in canine samples and for 48 h in equine and bovine samples.[41] In a study of canine and feline serum samples stored in capped plastic tubes, increases in pH and decreases in [fCa^{2+}] occurred by 1 h of storage at 24 °C, by 24 h at 4 °C, by 1 d at –20 °C, and by 30 d at –80 °C.[42] Normalized [fCa^{2+}] is reportedly stable in serum or plasma stored for 1 wk at 4 °C and for 6 wk at –20 °C;[35] such results should be similar to the actual [fCa^{2+}] in patients that lack significant acid-base and electrolyte disturbances.

C. Common clinical assays
1. [fCa^{2+}] is measured by direct potentiometry using Ca^{2+}-selective electrodes, typically in a blood gas analyzer or point-of-care analyzer with blood-gas capabilities. Only the active fraction of fCa^{2+} in the aqueous phase is assessed. The method actually measures the activity of the fCa^{2+} ions relative to aqueous solutions that by convention have ionic

strengths of 0.160 mol/kg, the assumed average ionic strength of blood's aqueous phase in health; the activity divided by an activity coefficient then yields a [fCa^{2+}].[43] Different activity coefficients for plasma Ca^{2+} and different sources of standard solutions could result in different measured [fCa^{2+}].

2. In disease states, sample factors may cause mild inaccuracies of [fCa^{2+}].
   a. Hypernatremia or hyponatremia: Marked hypernatremia increases plasma ionic strength, thus decreasing the activity coefficient for Ca^{2+}; when the measured activity is converted to a concentration using the analyzer's standard activity coefficient, the [fCa^{2+}] is falsely increased (about 0.4 mg/dL when the [Na^+] is 170 mmol/L). Marked hyponatremia decreases plasma's ionic strength, increases the activity coefficient of Ca^{2+}, and yields a false decrease in [fCa^{2+}] (e.g., by about 0.4 mg/dL when the [Na^+] is 110 mmol/L).[43] Testing other types of fluid samples with ionic strengths different from blood plasma would yield inaccurate results for [fCa^{2+}].
   b. Hyperlipidemia or marked hyperproteinemia: Marked increases in blood total solids occurring with lipemia or some paraproteinemias cause an overestimation of [fCa^{2+}]. An increase in the % total solids from 7 % to 15 % would falsely increase [fCa^{2+}] by about 0.5 mg/dL.[43]
   c. Whole blood sample hematocrit may mildly influence [fCa^{2+}] with some analyzers, results being greater with increasing hematocrit; errors were no more than 0.14 mmol/L (0.56 mg/dL) and usually much less.[44]
3. The [fCa^{2+}] of a sample may be reported as it was measured at the pH of the sample or as a *normalized [fCa^{2+}]*, which adjusts for pH-related interactions of fCa^{2+} with anions and is the [fCa^{2+}] that would be expected if the pH of the patient's sample were 7.4. If a normalized [fCa^{2+}] is reported, it should be noted as such. This value is sometimes called pH-adjusted, pH-corrected, or standardized [fCa^{2+}]. It should not be confused with the total Ca^{2+} concentrations adjusted for hypoalbuminemia or hypoproteinemia. Different formulas or factors have been used to calculate normalized [fCa^{2+}].
   a. Two equations for normalized [fCa^{2+}] are provided in Eq. 11.1 (terms have been modified for consistency). Eq. 11.1a was recommended by the Clinical and Laboratory Standards Institute;[45] Eq. 11.1b was derived for an instrument with a specific Ca^{2+}-selective electrode and a pH electrode.[46] Some veterinary laboratories apply their own empirical formula, which may be based on testing samples after acidification to adjust the pH into the analytical range of a blood gas analyzer. Note: Effects of in vitro versus in vivo pH changes may be somewhat different,[47] but the cause is generally unknown, and a single equation is applied.

$$\text{normalized } [fCa^{2+}] = \text{measured } [fCa^{2+}] \times 10^{(-0.24 \times (7.4 - pH))} \quad (\textbf{11.1a})$$

$$\text{normalized } [fCa^{2+}] = \text{measured } [fCa^{2+}] \times (1 + 0.53 \times (\text{actual pH} - 7.4)) \quad (\textbf{11.1b})$$

   b. Differences in the equations cause mildly different results for normalized [fCa^{2+}]. When interpreted, calculated patient values should be compared to RIs established by the same equation.
4. The normalized [fCa^{2+}] has advantages and disadvantages.
   a. If a patient is either acidemic or alkalemic, a normalized [fCa^{2+}] may be misleading because it does not represent the true [fCa^{2+}] in the blood.
   b. The pH adjustment removes the pH-dependent variations created by improper sample handling. For clinical interpretation, the normalized value must be compared to an appropriate normalized [fCa^{2+}] RI.

5. It has been recommended that the [fCa^{2+}] be reported with pertinent information: type of sample, site of collection, measured [fCa^{2+}], measured pH, and [fCa^{2+}] converted to a sample pH of 7.4.[35]

D. Unit conversion: mg/dL × 0.2495 = mmol/L, and mEq/L × 0.5 = mmol/L (SI unit)[31]

IV. General concepts for interpreting [tCa^{2+}] and [fCa^{2+}]

A. [fCa^{2+}] is tightly controlled by hormones and reflects the status of calcium homeostasis, whereas [tCa^{2+}] is unregulated and affected by changes in concentrations of proteins and NPA in addition to changes in [fCa^{2+}]. For example, [tCa^{2+}] may be decreased because of hypoproteinemia or hypoalbuminemia when [fCa^{2+}] is WRI and the animal does not have a defect in regulating the [fCa^{2+}]. Conversely, [fCa^{2+}] may be decreased or increased when [tCa^{2+}] is WRI, thus indicating abnormal Ca^{2+} regulation that may cause clinical signs or pathologic events. Therefore, it is an accurate [fCa^{2+}] rather than the [tCa^{2+}] that is of most interest when evaluating for disorders that affect Ca^{2+} homeostasis.

B. Because [fCa^{2+}] is affected by pH, accurate fCa^{2+} analysis requires a blood gas analyzer (as well as careful collection and processing) and is therefore not provided as part of a routine serum or plasma chemistry panel. Consequently, it is [tCa^{2+}] that is routinely measured and interpreted. Although [tCa^{2+}] often parallels [fCa^{2+}] and is a good screening test for disorders of Ca^{2+} homeostasis, [tCa^{2+}] does not necessarily reflect the more relevant [fCa^{2+}].

C. When interpreting a [tCa^{2+}], one must recognize the effect of lower protein and albumin concentrations on [tCa^{2+}]. Correction or adjustment formulas have been proposed to estimate the degree of effect, but they should be considered only rough approximations.

1. Canine adjusted [tCa^{2+}] considering [albumin] (Eq. 11.2a)[8]

$$\text{Canine adjusted } [tCa^{2+}] = \text{measured } [tCa^{2+}] - \text{measured [albumin]} + 3.5\ (\pm 1.3) \quad \textbf{(11.2a)}$$

Example: If [tCa^{2+}] = 8.0 mg/dL and [albumin] = 1.0 g/dL, then

Canine adjusted [tCa^{2+}] = 8.0 – 1.0 + 3.5 (±1.3) = 10.5 ± 1.3 = 9.2 to 11.8 mg/dL

Interpretation: If the dog were not hypoalbuminemic, its serum [tCa^{2+}] would be from 9.2 to 11.8 mg/dL in 95% of canine samples.

$$\text{Canine adjusted } [tCa^{2+}] = \text{measured } [tCa^{2+}] - (0.4 \times \text{measured [TP]}) + 3.3\ (\pm 1.6) \quad \textbf{(11.2b)}$$

Example: If [tCa^{2+}] = 8.0 mg/dL and [TP] = 4.0 g/dL, then

Canine adjusted [tCa^{2+}] = 8.0 – (0.4 × 4.0) + 3.3 (±1.6) = 9.7 ± 1.6 = 8.1 to 11.3 mg/dL

Interpretation: If the dog were not hypoproteinemic, its serum [tCa^{2+}] would be from 8.1 to 11.3 mg/dL in 95% of canine samples.

a. Failure to consider the "±1.3," which is an estimate of the 95 % confidence interval of the published data, promotes misinterpretations. The adjusted values are ballpark values, and the ballpark is big.

b. The formula should be used cautiously for three reasons: (i) the adjusted [tCa^{2+}] is at best an estimate because it does not consider nonprotein factors that affect [tCa^{2+}], (ii) the formula was generated using canine tCa^{2+} and albumin concentrations determined by one set of assays, and an identical formula would probably not be obtained if other assays were used, and (iii) the formula does not

consider the variations in Ca^{2+} binding to globulins. Ca^{2+} is bound to albumin and some globulins. The relative amount of Ca^{2+} bound to albumin and globulins varies with different albumin to globulin ratios, e.g., samples with panhypoproteinemia versus those with hypoalbuminemia and hyperglobulinemia.

2. Canine adjusted [tCa^{2+}] considering total protein concentration (Eq. 11.2b)
   a. Failure to consider the "±1.6," which is an estimate of the 95 % confidence interval of the published data, promotes misinterpretations.
   b. Even though the formula does include total protein concentration, it should be used cautiously for the aforementioned reasons.
3. Other investigators have found statistically significant correlations between [albumin] and [tCa^{2+}] in dogs, cats, horses, and cattle, but the correlations were weak, especially in cattle.[48,49] The general principle that [tCa^{2+}] varies with [albumin] is true, but the weakness and variation in the relationship among individuals indicates that rigid use of protein-correction formulas is not appropriate, especially in cattle.[7,50] Direct, proper measurement of [fCa^{2+}] is most informative. Consideration of protein concentrations when interpreting [tCa^{2+}] is helpful.

D. Acid-base status of an animal should be considered when interpreting Ca^{2+} results because it can affect the [fCa^{2+}] by three processes.
1. As explained in the analytical section, changing the blood pH affects the binding of Ca^{2+} to proteins and NPA. Acidemia increases the [fCa^{2+}] and alkalemia decreases the [fCa^{2+}].
2. When there is an organic acidosis, the organic ion (e.g., L-lactate, acetoacetate, or β-hydroxybutyrate) in the plasma may bind Ca^{2+} to lower the [fCa^{2+}], whereas concurrent acidemia would raise the [fCa^{2+}]. Also, the presence of the organic anion in renal tubular fluid would decrease the tubular resorption of Ca^{2+}. With these multiple factors, the actual alteration in [fCa^{2+}] may depend on the severity and duration of the organic acidosis.

E. With these concepts in mind, Figure 11.3 illustrates potential relationships of the three Ca^{2+} fractions. Major points that should be understood include the following:
1. An animal may have hypocalcemia because of hypoproteinemia or hypoalbuminemia, but the systems that regulate [fCa^{2+}] are not defective (Figure 11.3B).
2. If serum protein concentrations are WRI, then hypocalcemia typically indicates that the [fCa^{2+}] is decreased (Figure 11.3C).
3. Most hypercalcemic samples have an increased [fCa^{2+}] (Figure 11.3D).
4. Normocalcemia in the presence of hypoproteinemia suggests an increased [fCa^{2+}] (Fig 11.3E) or increased NPA.
5. The severity of decreased [fCa^{2+}] may not be evident from the [tCa^{2+}] if the concentrations of the bound-Ca^{2+} fractions are increased (Figure 11.3F).
6. The [fCa^{2+}] is affected by altered plasma pH and the presence of NPA in the plasma (Figure 11.3G).
7. Excess heparin (a NPA) in a blood sample can cause a falsely decreased [fCa^{2+}] (Figure 11.3H).

F. Some authors state that a "true hypocalcemia" or "true hypercalcemia" is present only when there is a decreased or increased [fCa^{2+}], respectively. Such terms are discouraged as they falsely imply that decreased or increased concentrations of tCa^{2+} are not truly hypocalcemia and hypercalcemia, respectively. Unfortunately, there is not a consistent method of referring to abnormal [fCa^{2+}]; "free hypocalcemia" and "free hypercalcemia" are suggested and used in this book, but others use "ionized hypocalcemia" and "ionized hypercalcemia" to describe decreases and increases in [fCa^{2+}], respectively. Such terms are

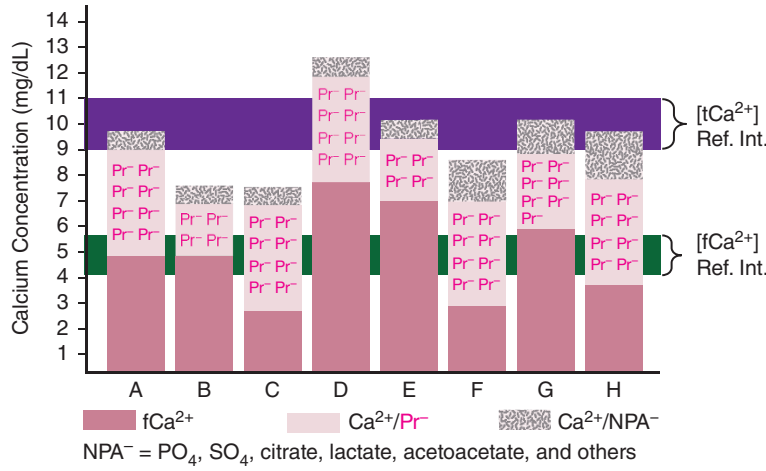


Figure 11.3. Conceptual relationships of total, free, and bound Ca^{2+} fractions in serum or plasma.

A. Healthy animal: The $[tCa^{2+}]$ and $[fCa^{2+}]$ are within respective RIs; the concentration of protein-bound Ca^{2+} is nearly 40 % of the $[tCa^{2+}]$, and the concentration of Ca^{2+} bound to NPAs is less than 10 %.

B. Hypoproteinemia (hypoalbuminemia): The ↓ $[tCa^{2+}]$ is caused by a ↓ [protein-bound Ca^{2+}]. The $[fCa^{2+}]$ (the regulated concentration) is WRI; there is not a Ca^{2+}-related disorder.

C. Primary hypoparathyroidism or hypovitaminosis D: The ↓ $[tCa^{2+}]$ is primarily caused by a ↓ $[fCa^{2+}]$ because of either inadequate PTH or vitamin D activity. In this schematic example, the bound-Ca^{2+} concentrations are unchanged. Similar findings could occur with furosemide-induced diuresis, pseudohypoparathyroidism, postparturient hypocalcemia, bovine grass tetany, and potentially ketonuria.

D. Primary hyperparathyroidism, HHM, humoral hypercalcemia of benign disorders, or hypervitaminosis D: The ↑ $[tCa^{2+}]$ is primarily caused by ↑ $[fCa^{2+}]$ because of increased activity of PTH, PTHrP, or vitamin D. In this schematic example, the bound-Ca^{2+} concentrations are unchanged. Hypoadrenocorticism may yield a similar pattern, but mild and likely with increased NPA-bound $[Ca^{2+}]$.

E. HHM and concurrent hypoproteinemia: The $[fCa^{2+}]$ is increased because of increased PTHrP activity, but the protein-bound Ca^{2+} concentration is decreased because of concurrent hypoproteinemia. The net result is a $[tCa^{2+}]$ WRI and an ↑ $[fCa^{2+}]$. A similar pattern could be found in other hypercalcemic disorders if there is concurrent hypoproteinemia.

F. Chronic renal failure (not horses): The $[fCa^{2+}]$ is mildly decreased because of the inadequate formation of $1,25(OH)_2D$. Concurrently, the concentration of Ca^{2+} bound to NPAs (e.g., Ca^{2+} bound to citrate or PO_4) is increased. In this schematic example, the protein-bound Ca^{2+} concentration is unchanged, and the net result is a mild hypocalcemia. The protein-bound Ca^{2+} concentration would be decreased with hypoalbuminemia caused by a protein-losing nephropathy.

G. Lactic acidosis variant: In this scenario, acidemia promotes Ca^{2+} detachment from proteins causing a ↓ in protein-bound $[Ca^{2+}]$ and an ↑ $[fCa^{2+}]$. Some of the released Ca^{2+} increased the Ca^{2+} bound to NPAs by binding to lactate. In this schematic example, the $[tCa^{2+}]$ is WRI but the protein-bound $[Ca^{2+}]$ is decreased. Multiple variations could occur because of multiple concurrent factors. This example illustrates that the $[tCa^{2+}]$ may be WRI when concentrations of each fraction are not.

H. Excess heparin in plasma: Collection of blood with an inappropriate amount of heparin (an anion) results in some of the fCa^{2+} binding to heparin and thus a ↓ $[fCa^{2+}]$ and ↑ NPA-bound $[Ca^{2+}]$. The $[tCa^{2+}]$ does not change.

useful when $[fCa^{2+}]$ is abnormal but $[tCa^{2+}]$ is WRI; in such a situation, the term normocalcemia would correctly describe $[tCa^{2+}]$ being WRI, but it might suggest normality when the increased or decreased $[fCa^{2+}]$ supports disturbed calcium homeostasis and a hypocalcemic or hypercalcemic disorder.

G. Interpretation of [fCa^{2+}] data in some publications is difficult because information relevant to interpretation is not provided. The following factors should be considered whenever one is interpreting or communicating [fCa^{2+}] results.
1. Was sample collection and handling adequate to ensure accurate results? More specifically, what steps were taken to prevent inaccuracies caused by excess heparin or exposure to air?
2. Was the reported [fCa^{2+}] a measured concentration or a normalized (pH-adjusted) concentration?
3. Are the terms hypercalcemia and hypocalcemia referring to [fCa^{2+}] or [tCa^{2+}]?

V. Hypercalcemia (↑ [tCa^{2+}]) and free hypercalcemia (↑ [fCa^{2+}]) (Table 11.2)
A. Increased Ca^{2+} mobilization from bone or absorption in intestine: These disorders can cause hypercalcemia and free hypercalcemia, but increased [tCa^{2+}] may not occur if there is concurrent hypoproteinemia and hypoalbuminemia.
1. Increased PTH or PTHrP activity
a. Primary hyperparathyroidism[51–53]
(1) Parathyroid adenomas or carcinomas secrete PTH that stimulates Ca^{2+} resorption from bone and increases Ca^{2+} absorption in the intestine. Hypercalcemia may be marked. Renal excretion of Ca^{2+} may be increased because free hypercalcemia leads to an increased amount of Ca^{2+} in the renal filtrate (more than can be resorbed even with increased PTH activity), but without enough urinary Ca^{2+} loss to prevent hypercalcemia and free hypercalcemia.
(2) Hypophosphatemia is expected because PTH is a potent phosphaturic agent. If GFR is decreased for any reason, there may be normophosphatemia or hyperphosphatemia because PTH cannot promote phosphaturia if PO_4 does not enter the tubular fluid.
(3) The [PTH] is expected to be WRI to increased. Of 210 hypercalcemic dogs with primary hyperparathyroidism, 73 % had a [PTH] WRI and 27 % had an increased [PTH].[54] In the presence of free hypercalcemia, an appropriate physiologic response would be a decreased [PTH]. Thus, a [PTH] that is WRI, particularly in the upper range of the RI concurrently with free hypercalcemia, indicates an inappropriate release of PTH.
b. HHM (pseudohyperparathyroidism) (Figure 11.4)
(1) In some paraendocrine neoplasms, a hypercalcemic agent is produced by cells that normally do not produce the agent. Nearly all neoplasms and especially carcinomas have the potential to be paraendocrine.
(2) Some paraendocrine neoplasms produce PTHrP. *PTHrP* is a polypeptide with the first 13 N-terminal amino acids being very similar to those of the N-terminus of PTH. It has bone osteoclastic and renal resorptive effects similar to PTH, and it can produce marked hypercalcemia. Many normal cells produce PTHrP, but very little of the PTHrP produced by these cells enters blood.[55] PTHrP plays important roles in fetal development, but PTH governs systemic Ca^{2+} homeostasis after birth.
(3) Neoplasms known to produce PTHrP (Note: Only some neoplasms of types associated with HHM cause hypercalcemia, and hypercalcemia without increased PTHrP does not exclude their presence.)

Table 11.2. Disorders and conditions that cause hypercalcemia or free hypercalcemia[a]

[tCa^{2+}]	[fCa^{2+}]	
↑	↑	Increased Ca^{2+} mobilization from bone or absorption in intestine
↑	↑	Increased PTH or PTHrP activity
↑	↑	Primary hyperparathyroidism (parathyroid neoplasms)
↑	↑	HHM:* lymphoma, carcinomas
↑	↑	Humoral hypercalcemia of benign disorders (see the text)
↑	↑	Increased vitamin D activity (hypervitaminosis D)
↑	↑	Exogenous sources of vitamin D
↑	↑	Rodenticides containing cholecalciferol
↑	↑	Compounds containing tacalcitol or calcipotriol
↑	↑	Plants containing vitamin D
↑	↑	Excess dietary intake of vitamin D
↑	↑	Endogenous sources of vitamin D
↑	↑	Granulomatous inflammation: blastomycosis, histoplasmosis, cryptococcosis, pulmonary angiostrongylosis
↑	↑	Neoplasm-associated hypervitaminosis D
↑	↑	Neoplasms in bone: myeloma, lymphoma, metastatic neoplasm
↑	↑	Lactulose administration (possibly)
		Decreased urinary excretion of Ca^{2+}
		Renal failure
↑	↑	Horses (common in both acute and chronic)*
↑	WRI-↑[b]	Dogs and cats (acute and chronic)
↑	↑	Hypoadrenocorticism in dogs and cats (other theories: increased Ca^{2+} bound to protein or citrate)
↑	↑	Ruptured urinary bladder in foals
↑	↑	Thiazide diuretics
↑	WRI	Increased protein-bound Ca^{2+}: hyperglobulinemia in multiple myeloma
		Other or unknown mechanisms
↑	↑	Idiopathic hypercalcemia in cats
↑	↑	Excess intravenous infusion of Ca^{2+}
↑	?	Hemoconcentration
↑	↑	Hypothyroidism (juvenile onset or primary acquired)
↑	↑	Retained fetus and endometritis in a dog

* A relatively common disorder, condition, or mechanism

[a] The arrows indicate which values can become increased with each disorder, condition, or mechanism.

[b] Effects on [fCa^{2+}] vary with the cause of renal failure.

Note: The [tCa^{2+}] in healthy young dogs (< 1 yr old, especially in large breeds) may be up to 2 mg/dL greater than the URL for [tCa^{2+}] in mature dogs.

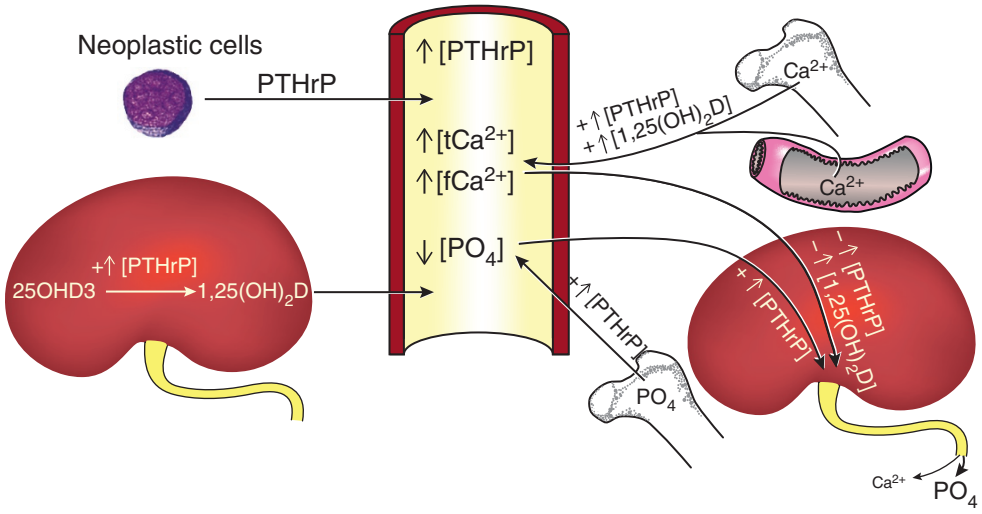


Figure 11.4. Humoral hypercalcemia of malignancy.
As represented by a lymphoma cell, various types of neoplastic cells (see text) can produce sufficient amounts of PTHrP to create hypercalcemia. PTHrP is a positive effector of 1α-hydroxylase and thus promotes increased formation of $1,25(OH)_2D$ in kidneys (not in horses). Increased PTHrP and $1,25(OH)_2D$ increase the mobilization of Ca^{2+} from bone and absorption of Ca^{2+} in intestine to lead to ↑ [fCa^{2+}] and typically ↑ [tCa^{2+}]. Increased PTHrP and $1,25(OH)_2D$ also reduce the renal excretion of Ca^{2+} by enhancing resorption of Ca^{2+} in the distal nephron. PTHrP also promotes PO_4 resorption from bone, but ↓ plasma [PO_4] is expected because of a greater effect on renal tubular cells to decrease PO_4 resorption and thus cause phosphaturia.

(a) In dogs, these neoplasms include lymphoma (usually T cell),[56,57] apocrine gland carcinoma,[57] other carcinomas (pulmonary, nasal, mammary, squamous cell, thyroid, thymic, clitoral, renal),[57–61] renal angiomyxoma,[62] and malignant melanoma.[63] Lymphoma is the most common neoplasm associated with hypercalcemia and free hypercalcemia in dogs, followed by apocrine gland adenocarcinoma of the anal sac.
(b) In cats, these neoplasms include pulmonary carcinoma, undifferentiated carcinoma, thyroid carcinoma, lymphoma,[64] and osteosarcoma.[65]
(c) In horses, myeloma and ameloblastoma have been reported to produce PTHrP.[66,67]
(d) An 8-yr-old cow with a mammary carcinoma had free hypercalcemia and hypophosphatemia that resolved with unilateral mastectomy; immunohistochemical labeling of the mass for PTHrP supported PTHrP production within the neoplasm.[68]

(4) Documentation of PTHrP involvement was limited by availability of valid assays for many years. Other hypercalcemia-inducing neoplasms that have been reported without documented increases in [PTHrP] include the following.
(a) In dogs, these neoplasms include epidermoid carcinoma[69] and metastatic adenocarcinoma.[70]
(b) In cats, these neoplasms include feline myeloproliferative disease,[71] squamous cell carcinoma,[72] and myeloma.[73]

(c) In horses, these neoplasms include gastric squamous cell carcinoma,[74,75] lymphoma,[76] plasma cell myeloma,[77] adrenocortical carcinoma,[78] and metastatic carcinoma.[79]

(5) Other expected findings

(a) Hypophosphatemia may be present. Its presence depends on the phosphaturic action of the agent relative to GFR. Decreased GFR reduces the amount of PO_4 that enters the tubular fluid and thus reduces the amount of PO_4 that is influenced by PTH activity.

(b) Increased [fCa^{2+}] should decrease PTH production[57] and yield undetectable to low-normal [PTH] in domestic mammals with HHM. Serum [PTH] determined by an N-terminal assay is often WRI (perhaps because nonfunctional PTH fragments are detected), but it is typically too low to suggest PTH-mediated hypercalcemia.

(c) Serum [PTHrP] should be increased if PTHrP is the hypercalcemic agent.

c. Humoral hypercalcemia of benign disorders

(1) In people, increased PTHrP production by benign neoplasms has been recognized and has been called *humoral hypercalcemia of benignancy*.[80] In dogs, evidence of such PTHrP-producing neoplasms (e.g., vaginal leiomyoma) has been rarely reported.[81]

(2) Two dogs with schistosomiasis caused by *Heterobilharzia americana* had hypercalcemia, normophosphatemia, increased [PTHrP], and decreased [PTH].[82] Necropsy failed to uncover a neoplasm, and it was concluded that the increased [PTHrP] was caused by the associated granulomatous inflammation, which is typically associated with macrophage production of $1,25(OH)_2D$. For the relatively few other dogs with schistosomiasis that have been hypercalcemic, [PTHrP] was not increased[83] or was not reported,[84] and $1,25(OH)_2D$ was not assessed.

2. Increased vitamin D activity (hypervitaminosis D)

a. Excessive vitamin D activity causes mild to marked hypercalcemia and free hypercalcemia through multiple processes.

(1) It stimulates formation of calbindin, a Ca^{2+}-binding protein, in intestinal mucosa so that more dietary Ca^{2+} is absorbed.

(2) It enhances bone resorption by promoting osteoclast activity.

(3) It promotes calbindin synthesis and Ca^{2+} resorption by renal tubular epithelial cells.

b. Hyperphosphatemia may be a concurrent finding because vitamin D promotes intestinal absorption of PO_4. However, $1,25(OH)_2D$ also stimulates production of FGF23, which promotes phosphaturia.[85,86]

c. Exogenous sources of vitamin D

(1) Ingestion of a rodenticide containing cholecalciferol (Quintox, Rampage, or Rat-Be-Gone), which is converted to 25OHD[87]

(2) Ingestion of tacalcitol or calcipotriol by dogs,[88,89] the latter (also known as calcipotriene) being present in a topical ointment (Dovonex, Sorilux) that is used for human psoriasis

(3) Ingestion of plants containing various forms of vitamin D including $1,25(OH)_2D3$ in *Cestrum diurnum* (day-blooming jessamine) in Florida[90] and *Solanum* spp. or *Nierembergia* spp. in Hawaii or parts of South America[55,91,92]

(4) Excess dietary intake of vitamin D,[93–97] including ingestion of misformulated commercial feeds and potentially salmon oil supplements[98,99]

d. Endogenous sources of vitamin D
(1) Granulomatous inflammation
(a) Hypercalcemia and free hypercalcemia have been reported in domestic mammals with a variety of infectious diseases including blastomycosis,[100] histoplasmosis,[101] cryptococcosis,[102] and pulmonary angiostrongylosis.[103] Noninfectious granulomatous inflammation (e.g., related to foreign material or fat) might also cause hypercalcemia through this mechanism.[104,105]
(b) The hypercalcemia is thought to occur when histiocytes or macrophages produce sufficient $1,25(OH)_2D$, but the mechanism of hypercalcemia is often not proven.
(2) Neoplasm-associated hypervitaminosis D: Inappropriately high $1,25(OH)_2D$ concentrations have been reported with canine apocrine gland adenocarcinoma and lymphoma.[57] The neoplastic cells may be producing $1,25(OH)_2D$ or stimulating $1,25(OH)_2D$ synthesis in other cells.

3. Neoplasms (hemic or nonhemic) in bone (not humoral hypercalcemia)
a. Hypercalcemic agents other than PTH, PTHrP, or vitamin D have been associated with malignancies in bone. Some dogs with lymphoma and myeloma have localized bone resorption and corresponding hypercalcemia and free hypercalcemia.[55] The hypercalcemic agents may be working locally or systemically to promote osteoclastic activity or osteoclastogenesis; they may include IL-1, IL-6, tumor growth factor, TNF, and prostaglandins.[55,57]
b. The [Pi] may be WRI or increased.

4. Lactulose administration has been the suspected cause of hypercalcemia in dogs and cats based on association with its use and its reported effect on intestinal cation absorption.[106] Lactulose reaches the distal intestine undigested and undergoes bacterial fermentation, which generates acids that promote Ca^{2+} and Mg^{2+} absorption.[107,108]

B. Decreased urinary excretion of Ca^{2+}: Unless stated otherwise, these disorders can cause hypercalcemia and free hypercalcemia, but an increased [tCa^{2+}] may not occur if there is concurrent hypoproteinemia and hypoalbuminemia.
1. Renal failure
a. Horses with some acute or chronic renal diseases
(1) In health, equine kidneys excrete excess dietary Ca^{2+}. Renal diseases that decrease GFR impair renal excretion of Ca^{2+} and thus cause or contribute to hypercalcemia and free hypercalcemia.[109] Lowering dietary Ca^{2+} intake by switching from alfalfa hay to grass hay can reduce or eliminate the hypercalcemia, but the impaired GFR persists.[110] Alfalfa hay can contain 2–10 times the Ca^{2+} content of grass or mixed hay.[111]
(2) Hypophosphatemia may be present.
(3) Horses with hypercalcemic renal failure have an expected decrease in [PTH].[112]
b. Dogs and cats with acute or chronic renal disease; hypercalcemia may occur with or without free hypercalcemia
(1) A minority of dogs and cats with acute renal failure are hypercalcemic, and in some, renal failure may be secondary to hypercalcemia caused by a different disorder. An increased concentration of Ca^{2+} bound to citrate or PO_4 may contribute to the increased [tCa^{2+}]. Raisin and grape (*Vitis* spp.) toxicoses in dogs frequently result in hypercalcemic acute renal failure; mechanisms other than Ca^{2+} binding to NPA may be involved.[113]

(2) Dogs: Of dogs with chronic renal failure, 10–15 % are reported to be hypercalcemic, and some have increased [fCa^{2+}].[114] Binding of Ca^{2+} to retained anions contributes to increased [tCa^{2+}].[115] The increase in [fCa^{2+}] has not been explained; decreased renal excretion may contribute. However, most dogs with chronic renal failure have a low-normal to mildly decreased [tCa^{2+}]. Binding of Ca^{2+} to retained anions may also yield normocalcemia ([tCa^{2+}] WRI) that masks free hypocalcemia.

(3) Cats: Increased [tCa^{2+}] has been reported in about 4–20 % of cats with chronic renal failure, and [fCa^{2+}] has been increased, usually mildly, in 10–30 % of them.[116] Evidence suggests that hypercalcemia is not mediated by PTH or vitamin D. Potential contributors include reduced renal excretion of Ca^{2+}, concurrent unassociated and unrecognized hypercalcemic disease, patient management using a PO_4-restricted renal diet with a relatively high Ca^{2+}:PO_4 ratio,[117] assay imprecision, preanalytical or analytical errors, and inappropriate RIs.

2. Hypoadrenocorticism (Addison's disease) in dogs and cats
   a. About 30 % of dogs with hypoadrenocorticism are hypercalcemic.[55,118] Hypercalcemia has also been described in cats with hypoadrenocorticism.[119,120] The reason for the hypercalcemia is not established but probably is at least partially caused by decreased renal excretion. The [fCa^{2+}] is increased in some affected dogs,[114,121,122] but [PTH], [PTHrP], and [1,25$(OH)_2$D] were mostly WRI ([PTH] was decreased or in the lower quartile of the RI in four of five dogs), so these mediators do not appear responsible for the hypercalcemia.[121,122] Increased Ca^{2+} binding to protein or citrate may contribute to increased [tCa^{2+}].
   b. Renal Ca^{2+} excretion in adrenalectomized dogs is decreased because of increased tubular resorption of Ca^{2+}.[123] The reason for enhanced tubular resorption is not established, but it may involve angiotensin II or cortisol deficiency.
      (1) When dogs with hypoadrenocorticism become hypovolemic because of vomiting, diarrhea, or impaired renal concentrating ability, angiotensin II activity increases. Angiotensin II promotes Na^+ and H_2O resorption in proximal renal tubules, thereby promoting passive Ca^{2+} resorption down the resulting concentration gradient. Thus, enhanced resorption of Na^+ may also promote the proximal tubular resorption of Ca^{2+} and cause hypercalcemia and free hypercalcemia. Concurrent hemoconcentration itself may slightly increase the serum [tCa^{2+}].
      (2) Administration of glucocorticoids increases renal excretion of Ca^{2+}. Thus, a deficiency in cortisol may allow more Ca^{2+} to be resorbed.
3. Ruptured urinary bladder in foals: These foals may develop hypercalcemia (authors' unpublished data), but most do not. When present, the hypercalcemia is probably caused by the resorption of urinary Ca^{2+} from the peritoneal cavity.
4. Thiazide diuretics: These act in the distal nephron to promote hypernatruria and, secondarily, volume depletion. Hypovolemia promotes enhanced proximal tubular resorption of Na^+ and, secondarily, passive proximal tubular resorption of Ca^{2+}.[124] Thiazides also promote the resorption of Ca^{2+} in the distal tubules. This form of hypercalcemia is rarely reported in domestic animals but does occur in dogs.[125] Thiazide diuretics may unmask subclinical hyperparathyroidism, and some studies in dogs suggest they might cause parathyroid gland hyperplasia.[126]

C. Increased protein-bound Ca^{2+}: In some cases of marked hyperproteinemia associated with a plasma cell myeloma, there is an increase in negatively charged globulins that bind cations including Ca^{2+}.[127] When fCa^{2+} associates with proteins, the [fCa^{2+}] transiently decreases and there is compensatory release of PTH to increase [fCa^{2+}]. The net result is an increased [tCa^{2+}] and a [fCa^{2+}] WRI.

D. Other or unknown mechanisms

1. Idiopathic hypercalcemia in cats:[128,129] These cats have persistent (months) hypercalcemia and free hypercalcemia, which may be marked, with no recognized hypercalcemic disorder. Typically, [PTH] is in the lower half of the RI, [PTHrP] is not increased, and [25OHD] and [$1,25(OH)_2D$] are low or WRI, indicating that these substances are not causing the hypercalcemia. Clinical signs are generally mild except for those caused by calcium oxalate urolithiasis, which develops in some affected cats.
2. Excess intravenous infusion of Ca^{2+}: This can be a mechanism of hypercalcemia and free hypercalcemia if the rate of administration exceeds the rate of renal excretion.
3. Hemoconcentration: If hypercalcemia occurs, expect it to be mild and associated with increased protein- or NPA-bound Ca^{2+}. It may also be related to angiotensin II stimulating resorption of Na^+ and Ca^{2+} in proximal tubules.
4. Hypothyroidism (juvenile onset[130] and primary acquired):[131] Hypercalcemia may be caused by increased intestinal absorption and decreased renal excretion of Ca^{2+}.
5. Retained fetus and endometritis in a dog[132]

VI. Hypocalcemia (↓ [tCa^{2+}]) and free hypocalcemia (↓ [fCa^{2+}]) (Table 11.3): Unless stated otherwise, these disorders can cause hypocalcemia and free hypocalcemia, but [tCa^{2+}] may not be decreased if there are concurrent increases in protein- or NPA-bound Ca^{2+}.

A. Hypoalbuminemia (hypoproteinemia)[8]

1. Hypocalcemia results from a decreased concentration of negatively charged proteins and therefore of protein-bound Ca^{2+}. The regulation of the [fCa^{2+}] would be adequate in these animals unless there is a concurrent defect in the regulation of [fCa^{2+}].
2. It has been called *pseudohypocalcemia* because the [fCa^{2+}] does not decrease and clinical signs of hypocalcemia do not occur. However, the animal truly has hypocalcemia (less Ca^{2+} per plasma volume than expected in health) when the [tCa^{2+}] is lower than the appropriate RI.

B. Inadequate Ca^{2+} mobilization from bone or absorption in intestine

1. Decreased PTH activity
   a. Primary hypoparathyroidism
      (1) Parathyroid glands are damaged (by trauma, surgery, neoplasia, or inflammation) or hypoplastic (after excision of a parathyroid adenoma that has suppressed the remaining glands) and do not respond adequately to free hypocalcemia or do not release adequate quantities of PTH to prevent free hypocalcemia. In 12 canine cases in which there was tissue analysis, the presence of lymphocytic parathyroiditis suggested an immune-mediated disease.[133]
      (2) Hypocalcemia, which may be marked, is caused by decreased resorption of Ca^{2+} from bone and/or decreased Ca^{2+} absorption in the intestine. Renal excretion of Ca^{2+} should be increased initially because of decreased PTH-stimulated resorption, but it may decrease due to less fCa^{2+} entering the renal filtrate when hypocalcemia is present.

Table 11.3. Disorders and conditions that cause hypocalcemia or free hypocalcemia[a]

[tCa^{2+}]	[fCa^{2+}]	
↓	WRI	Hypoalbuminemia (hypoproteinemia)*
↓	↓	Inadequate Ca^{2+} mobilization from bone or absorption in intestine
↓	↓	Decreased PTH activity
↓	↓	Primary hypoparathyroidism (damaged parathyroid gland)
↓	↓	Pseudohypoparathyroidism (decreased PTH receptor responsiveness)
↓	↓	Hypomagnesemia (bovine grass tetany)
↓	↓	Secondary hypoparathryoidism after removing hypercalcemic stimulus
↓	↓	Decreased vitamin D activity (hypovitaminosis D)
↓	↓	Chronic or acute renal failure in dogs, cats, and cattle*
↓	↓	Protein-losing nephropathy
↓	↓	Protein-losing enteropathy or chronic enteropathy in dogs
↓	↓	Dietary vitamin D deficiency (rare)
↓	↓	Hereditary $1,25(OH)_2D$–resistant rickets (vitamin D–dependent rickets, type II)
↓	↓	Vitamin D–dependent rickets type Ib
↓	↓	Exocrine pancreatic insufficiency (dogs)
↓	↓	Parturient, lactational, and preparturient hypocalcemia (milk fever, puerperal tetany in dogs, preparturient hypocalcemia in queens)*
↓	↓	Hypercalcitoninism: thyroid C-cell neoplasm, iatrogenic (calcitonin therapy)
↓	↓	Nutritional hypocalcemia (nutritional secondary hyperparathyroidism) (rare)
↓	↓	Oxalate toxicity
↓	↓	Bisphosphonate administration (e.g., pamidronate, alendronate)
↓	↓	Excess urinary excretion of Ca^{2+}
↓	↓	Ethylene glycol toxicosis (dogs and cats)
↓	↓	Metabolic alkalosis
↓	↓	Furosemide treatment
		Ca^{2+} binding with diffusible anions
WRI[b]	↓	Ca^{2+}-binding anticoagulants: EDTA,[b] citrate, oxalate (in vivo or in vitro)
WRI	↓	Tetracycline administration
See text		Ca^{2+} deposition during fracture healing
		Other or unknown mechanisms
↓[c]	↓	Endotoxemia, sepsis, and other critical illness
↓[c]	↓	Acute pancreatitis (dogs and cats)*
↓[c]	↓	Urinary tract obstruction*
↓	↓	PO_4 enemas
↓	↓	Diabetic ketoacidosis in dogs and cats
↓	↓	Blister beetle poisoning (cantharidiasis)

Table 11.3. Disorders and conditions that cause hypocalcemia or free hypocalcemia[a] (Continued)

$[tCa^{2+}]$	$[fCa^{2+}]$	
↓	↓	Myopathies (see the text)
↓	↓	Acute tumor lysis syndrome
↓	↓	Grain overload (acute carbohydrate ruminal engorgement)
WRI	↓	Hyperthyroid cats
WRI	↓	Endurance events (horses)

* A relatively common disorder, condition, or mechanism

[a] A ↓ $[fCa^{2+}]$ without a ↓ $[tCa^{2+}]$ is not hypocalcemia with respect to $[tCa^{2+}]$, but it is hypocalcemia of fCa^{2+} (free hypocalcemia). Arrows indicate which values can become decreased with each disorder, condition, or mechanism.

[b] ↓ $[tCa^{2+}]$ if EDTA provides the anion

[c] $[fCa^{2+}]$ is decreased more consistently than $[tCa^{2+}]$.

Note: The $[tCa^{2+}]$ may be falsely decreased if the sample contains EDTA (wrong tube or contamination of clot tube). A concurrent false hyperkalemia would be expected with the potassium salt of EDTA.

(3) The serum [PTH] is decreased or low-normal. In contrast, with free hypocalcemia and functional parathyroid glands, the [PTH] should be increased as a physiologic response.

(4) Hyperphosphatemia is expected because of increased resorption of PO_4 by renal tubules (because of less PTH to promote phosphaturia), and thus renal excretion of PO_4 is decreased. However, renal excretion of PO_4 may increase later because hyperphosphatemia causes an increased amount of PO_4 to pass into the renal filtrate or because PO_4 stimulates production of FGF23, a phosphaturic agent.[85,86]

(5) Hyperphosphatemia enhances hypocalcemia by inhibiting renal formation of $1,25(OH)_2D$ (except in horses).

b. Pseudohypoparathyroidism

(1) This term refers to a group of rare genetic disorders in which PTH receptors or post-PTH receptor pathways are unresponsive. Therefore, free hypocalcemia develops because of diminished PTH effects on intestine, bone, and kidneys.

(2) The [Pi] may be increased because of inadequate renal excretion of PO_4. The [PTH] should be increased because of the free hypocalcemia.

(3) In veterinary medicine, the disorder has been suspected in a ferret.[134]

(4) The term *pseudohypoparathyroidism* also has been used for a decreased responsiveness to PTH in dairy cows with milk fever.[135]

c. Hypomagnesemia

(1) Hypomagnesemia caused by Mg^{2+} depletion may produce a functional hypoparathyroid state and hypocalcemia. The dysfunction may be due to two processes:

(a) Mg^{2+}-depleted cells may be resistant to PTH. PTH cellular responses are mediated by adenylate cyclase, which requires Mg^{2+}.

(b) Parathyroid cells secrete less PTH either because of diminished cyclic adenosine monophosphate (cAMP) formation (impaired adenylate

cyclase) or because of diminished competitive inhibition of Ca^{2+} binding to parathyroid cells.[136] In the presence of hypomagnesemia, there may be more available binding sites for Ca^{2+} on the parathyroid cells and thus a lesser [fCa^{2+}] is able to inhibit PTH secretion.[137]

(2) These mechanisms may explain the hypocalcemia of grass tetany in cattle and free hypocalcemia in dogs with protein-losing enteropathy and marked hypomagnesemia.[138]

d. Secondary hypoparathyroidism after removing an underlying hypercalcemic stimulus: This has been suspected in a dog that became transiently hypocalcemic after treatment for lymphoma[139] and in another dog after treatment of a metastatic adenocarcinoma of the anal sac.[140]

2. Decreased vitamin D activity (hypovitaminosis D)

a. Chronic renal disease or failure in dogs, cats, and cattle

(1) Dogs, cats, and cattle with chronic renal disease may have a low-normal [tCa^{2+}] or mild hypocalcemia because of abnormal vitamin D metabolism. In compensating for hypocalcemia or low $1,25(OH)_2D$ concentration, dogs, cats, and cattle develop secondary hyperparathyroidism.[141] Similar changes are not expected in horses because equine kidneys lack 1α-hydroxylase activity;[17] decreased GFR in horses tends to cause hypercalcemia, not hypocalcemia.

(2) Steps in the development and progression of secondary renal hyperparathyroidism are shown in Figure 11.5. Details of its pathogenesis are illustrated in Figure 11.6.

(3) Three contributions to the pathologic state are not illustrated in Figure 11.6.

(a) A decrease in the number of vitamin D receptors in the parathyroid glands creates a state of vitamin D resistance.[143,144] With less vitamin D inhibition and decreased responsiveness to FGF23, the cells of the parathyroid gland proliferate and produce more PTH.

(b) Megalin, a renal tubular receptor for the complex of 25OHD and vitamin-D-receptor protein, is reduced in chronic renal disease. Less uptake of the complex from the glomerular filtrate contributes to 25OHD deficiency and decreased renal $1,25(OH)_2D$ formation, thus causing a decreased [$1,25(OH)_2D$].[143]

(c) Increased [FGF23] contributes to a 25OHD deficiency and therefore decreased [$1,25(OH)_2D$] by enhancing 24-hydroxylase activity, which catalyzes conversion of 25OHD and $1,25(OH)_2D$ to $24,25(OH)_2D$ and $1,24,25(OH)_3D$, respectively (Figure 11.11).[143]

(4) Other mechanisms that may contribute to hypocalcemia in chronic renal failure include clearance of Ca^{2+} complexed with PO_4, formation of Ca^{2+}-PO_4 complexes in tissue, and skeletal resistance to increased [PTH].

b. Acute renal failure: When hypocalcemia and free hypocalcemia occur in animals with acute renal failure, the cause of the hypocalcemia may be either the renal failure or a concurrent pathologic state such as acute pancreatitis, ethylene glycol poisoning, or hypoalbuminemia.[149] As with chronic renal failure, hypocalcemia may be promoted by decreased renal formation of $1,25(OH)_2D$, clearance of Ca^{2+} complexed with PO_4, formation of Ca^{2+}-PO_4 complexes in necrotic renal tissue, skeletal resistance to increased [PTH], and cytokine-induced upregulation of CaSRs on parathyroid and renal tubular epithelial cells.[150]

c. Protein-losing nephropathy: In addition to the above factors that contribute to lower [25OHD] and [$1,25(OH)_2D$], proteins lost due to glomerular disease include

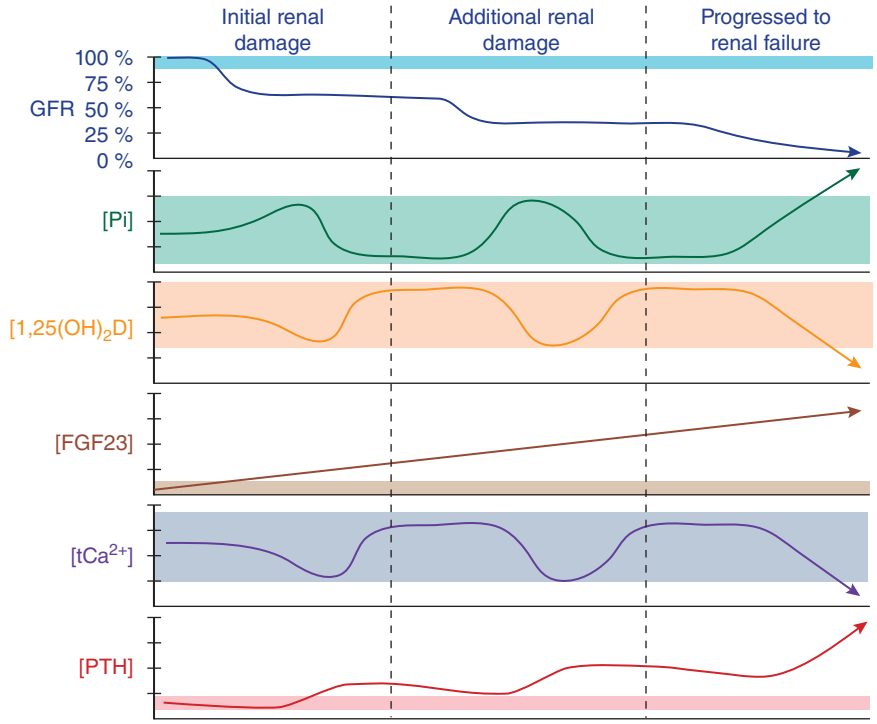


Figure 11.5 Schematic pathogenesis of secondary renal hyperparathyroidism in dogs, cats, and cattle. *Shaded areas* represent RIs for GFR or each analyte concentration.

- Initial renal damage: The sequence of events detailed in Figure 11.6 initially compensates for decreased clearance of PO_4 and inadequate formation of $1,25(OH)_2D$. A new homeostasis in Ca^{2+} and PO_4 balance is maintained by ↑ PTH secretion and ↑ [FGF23].
- Additional renal damage: As renal disease progresses and more nephrons are lost, pathophysiologic responses recur that stimulate more PTH and FGF23 synthesis, thus helping to maintain physiologic concentrations of fCa^{2+}, PO_4, and $1,25(OH)_2D$.
- Progressed to renal failure: Eventually, renal disease reduces GFR sufficiently for serum [Pi] to remain increased, and insufficient $1,25(OH)_2D$ and PTH are made to maintain $[fCa^{2+}]$. The animal is presented with clinical signs of renal insufficiency or failure, azotemia, impaired ability to concentrate or dilute urine, mild hypocalcemia, and hyperphosphatemia. The $[tCa^{2+}]$ may not reflect the abnormal regulation of the $[fCa^{2+}]$ because of the Ca^{2+} that is bound to NPAs that are not excreted in renal failure.

VDBP, the major carrier protein of 25OHD and $1,25(OH)_2D$. The renal loss of VDBP may contribute to decreased [25OHD] and $[1,25(OH)_2D]$, thus impacting calcium homeostasis (see Vitamin D section).

d. Protein-losing enteropathy or chronic enteropathy in dogs:[151,152] Dogs with lymphangiectasia and lymphocytic or plasmacytic enteritis can have concurrent hypocalcemia, free hypocalcemia, decreased [25OHD], and decreased $[1,25(OH)_2D]$. Decreased [25OHD] occurred in dogs with chronic inflammatory bowel disease whether or not they had evidence of protein-losing enteropathies.[153] Decreases in vitamin D concentrations may be due to decreased intake of food,

decreased absorption of vitamin D, or loss of vitamin D or its plasma carriers (e.g., albumin). Dogs with chronic enteropathies and decreased [25OHD] commonly had increased [PTH], an appropriate response to hypocalcemia (secondary hyperparathyroidism).[154] However, many affected dogs also have low [fMg^{2+}], and concurrent severe hypomagnesemia may rarely cause functional hypoparathyroidism that contributes to hypocalcemia.[138]

e. Dietary vitamin D deficiency: It has been induced experimentally[155,156] and may occur clinically.[157]

3. Hereditary $1,25(OH)_2D$–resistant rickets (vitamin D–dependent rickets, type II) (rare)
   a. The clinical manifestations of hereditary $1,25(OH)_2D$–resistant rickets are produced by defective target-organ receptors and response to vitamin D (primarily $1,25(OH)_2D$). The defective intestinal absorption of Ca^{2+} and defective responses in bone and kidneys cause free hypocalcemia, a compensatory increase in [$1,25(OH)_2D$], and secondary hyperparathyroidism (with increased [PTH]).
   b. A young cat that had this disorder had hypocalcemia (3.9 mg/dL), increased [PTH], and increased [$1,25(OH)_2D$].[158] It was presented because of vomiting, diarrhea, muscle tremors, and mydriasis.
   c. A mutation in the vitamin D receptor gene was identified in a young Pomeranian with nonpruritic alopecia, gross and radiographic limb abnormalities, exercise intolerance, and intermittent lameness. She had severe hypocalcemia, decreased [25OHD], and secondary hyperparathyroidism. The hypocalcemia did not correct with calcitriol ($1,25(OH)_2D$) therapy. The authors concluded that either the vitamin D receptor was not expressed, or it could not bind $1,25(OH)_2D$.[159]
4. Vitamin D–dependent rickets type Ib (rare): An affected 3-mo-old cat that was presented because of seizures and reluctance to move had a mutation in the vitamin D 25-hydroxylase gene (*CYP2R1*) resulting in total and free hypocalcemia, increased [PTH], and decreases in [25OHD] and [$1,25(OH)_2D$].[160]
5. Exocrine pancreatic insufficiency (dogs)
   a. Idiopathic pancreatic acinar atrophy, chronic pancreatitis, pancreatic neoplasia, or pancreatic surgery may lead to exocrine pancreatic insufficiency and thus cause incomplete digestion of dietary lipids and other ingesta (see Chapter 15).
   b. Dietary Ca^{2+} may be less available for intestinal absorption because of its binding to partially digested lipids. Also, maldigestion of lipids may reduce absorption of fat-soluble vitamins, including vitamin D.
   c. Many dogs with exocrine pancreatic insufficiency develop hypoalbuminemia that also would contribute to hypocalcemia.
6. Parturient, lactational, and preparturient hypocalcemia
   a. Parturient hypocalcemia (milk fever) in cattle: Hypocalcemia, which may be marked, results from excess Ca^{2+} loss (via milk or fetal bone development) relative to intestinal absorption and bone resorption. Because most paretic cattle have an increased [PTH], the disorder involves an inadequate response of target cells (e.g., decreased receptors or defective biochemical pathways); [$1,25(OH)_2D$] may be increased, but to an attenuated degree.[161,162] Dietary factors that promote metabolic alkalosis and hypomagnesemia may decrease the target cell response to PTH.[135,163] Free hypocalcemia leads to clinical signs (hyperesthesia and tetany early, and paresis to flaccid paralysis later) because of defective neural transmissions and muscle contractions. Hypophosphatemia may occur because of lactation, poor bone resorption, and the phosphaturic action of PTH.

b. Puerperal tetany (eclampsia) in bitches, mares, and ewes:[164] Mild to marked hypocalcemia is associated with peak lactation and corresponding peak loss of Ca^{2+} in milk.[165]
c. Preparturient hypocalcemia in queens:[166] In a report of four cats, clinical signs (anorexia, depression, and lethargy) occurred 3–17 d prior to parturition. Three of the queens had a decreased [tCa^{2+}]. The [fCa^{2+}] was decreased in the one cat in which it was measured.

7. Hypercalcitoninism
   a. This is usually caused by thyroid C-cell neoplasms (medullary thyroid carcinomas), which are relatively uncommon neoplasms reported most often in older bulls.[167] When associated with C-cell neoplasms, hypercalcitoninism usually causes only mild hypocalcemia because of the following:
      (1) Neoplasms are seen in older animals, and their bones are relatively refractory to calcitonin (calcitonin does not inhibit PTH-stimulated osteoclastic activity).
      (2) Lowering of the [fCa^{2+}] causes a compensatory increase in PTH production that helps maintain a physiologic [fCa^{2+}].
   b. Marked hypocalcemia may occur if a C-cell neoplasm destroys parathyroid glands, which causes hypoparathyroidism in addition to hypercalcitoninism.
   c. Excess administration of salmon calcitonin (to lower the [tCa^{2+}] in hypervitaminosis D or HHM) may cause hypocalcemia.
8. Nutritional hypocalcemia (nutritional secondary hyperparathyroidism)
   a. This occurs with vitamin D–deficient diets, when diets are not balanced for Ca^{2+} and PO_4 (i.e., the dietary Ca^{2+}:PO_4 ratio is lower than the desired ratio for the species), or when these problems are concurrent.[157] The diet causes the free hypocalcemia, which stimulates parathyroid gland hyperplasia and release of PTH, thus promoting increased [$1,25(OH)_2D$] if the diet is not deficient in vitamin D. Imbalanced diets may include excessive PO_4 (carnivores' meat diet)[168] and/or a relative or absolute deficiency of Ca^{2+}. The net effect is too much PO_4 and too little Ca^{2+} in the body.
   b. A decrease in the [fCa^{2+}] stimulates parathyroid glands to cause secondary hyperparathyroidism. The increased PTH release tends to keep the [fCa^{2+}] and [tCa^{2+}] WRIs or near RIs at the expense of the Ca^{2+} content of bones, and thus fibrous osteodystrophy may develop, especially in young animals (the bones of older animals are relatively refractory to PTH-stimulated osteoclastic activity). The degree of hypocalcemia depends on the severity of dietary imbalance and the availability of Ca^{2+} from bones (with Ca^{2+} being more available in young animals, initially).
   c. Renal PO_4 excretion is increased because of the phosphaturic actions of PTH and, in some cases, the high dietary intake of PO_4. If the renal PO_4 excretion is measured, one should expect increased 24-h PO_4 clearance and increased fractional excretion of PO_4.
9. Oxalate toxicity[169]
   a. Horses that eat plants that have high oxalate content but low Ca^{2+} content absorb less dietary Ca^{2+} and develop hypocalcemia and free hypocalcemia. Such plants include buffel grass (*Cenchrus ciliaris*), pangola (*Digitaria* spp.), *Setaria*, kikuyu (*Pennisetum clandestinum*), rhubarb (*Rheum rhabarbarum*), halogeton (*Halogeton glomeratus*), greasewood (*Sarcobatus* spp.), and soursob (*Oxalis pes-caprae*).
   b. In ruminants, halogeton and curly dock (*Rumex crispus*) may cause hypocalcemia and free hypocalcemia.[170]

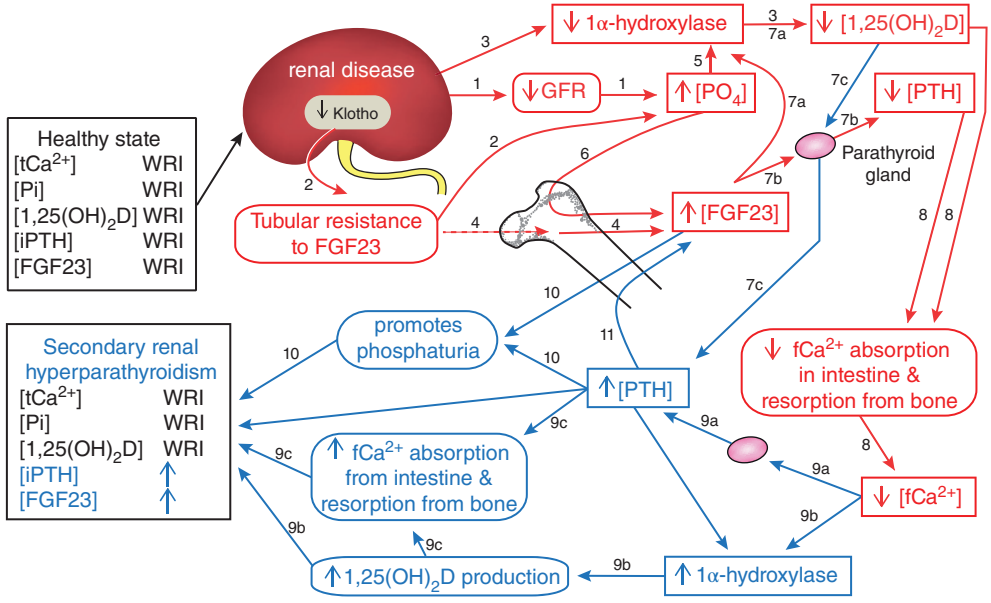


Figure 11.6. Sequential events during the development of secondary renal hyperparathyroidism caused by chronic renal disease in dogs, cats, and cattle. Eventually, tCa^{2+}, Pi, and $1,25(OH)_2D$ concentrations may be abnormal (see Figure 11.5).

Factors that lead to ↓ [fCa^{2+}] (red; arrow numbers 1–8 correspond to steps below)

- Renal disease causes:
  1. A progressive decrease in GFR (due to a loss of nephrons), which causes less urinary excretion of PO_4, so mild hyperphosphatemia (measured and reported as [Pi]) develops
  2. A progressive decrease in cellular Klotho due to fewer tubular cells and downregulation, which causes tubular resistance to FGF23 and promotes tubular resorption of PO_4 (thus an increase in [PO_4])
  3. A decrease in 1α-hydroxylase activity due to decreased Klotho and fewer nephrons, which causes less formation of $1,25(OH)_2D$ from 25OHD
  4. Increased FGF23 formation by osteocytes prior to hyperphosphatemia (mechanism unclear), later promoted by hyperphosphatemia, and increased renal renin production (not shown) secondary to lower [$1,25(OH)_2D$] (see step 5); activation of the renin–angiotensin–aldosterone system reduces Klotho expression, thus increasing FGF23 formation[142]
- A greater [PO_4] causes:
  5. Decreased 1α-hydroxylase activity, which causes less formation of $1,25(OH)_2D$ from 25OHD
  6. Increased production of FGF23 by osteocytes
  7. An increase in [FGF23], which in turn causes:
     a. Decreased 1α-hydroxylase activity and therefore less formation of $1,25(OH)_2D$ from 25OHD and a lower plasma [$1,25(OH)_2D$]
     b. Decreased production and release of PTH and a lower plasma [PTH]
     c. A stimulus for increased production of PTH because of lower [$1,25(OH)_2D$] (more important later in renal failure, see below)
  8. The resulting lower PTH and 1,25(OH)2D concentrations lead to decreased Ca^{2+} absorption in intestine and resorption from bone, and eventually a lower [fCa^{2+}].

c. Alfalfa has high oxalate content, but ingestion does not cause hypocalcemia because alfalfa also has high Ca^{2+} content.

10. Bisphosphonate administration (e.g., pamidronate, alendronate) inhibits bone resorption, thus promoting hypocalcemia, free hypocalcemia, hypophosphatemia, and hypomagnesemia.[171] Free hypocalcemia may be more likely in patients with underlying hormonal disturbances in calcium homeostasis.

C. Excess urinary excretion of Ca^{2+}

1. Ethylene glycol toxicosis (dogs and cats)
   a. Products of ethylene glycol metabolism (including oxalates) bind Ca^{2+} in renal tubular fluid and thus cause hypercalciuria. Also, a high plasma oxalate concentration may cause intravascular formation of calcium oxalate crystals.[172]
   b. Acute nephrosis may develop, causing decreased tubular resorption of Ca^{2+}.[173,174]
2. Metabolic alkalosis
   a. If an animal has a persistent metabolic alkalosis resulting in a plasma [HCO_3^-] above the renal threshold for HCO_3^-, the resultant bicarbonaturia would obligate the renal loss of cations such as Ca^{2+}.
   b. Intravenous $NaHCO_3^-$ infusions in cats:[175] Both the [tCa^{2+}] and [fCa^{2+}] were reported to be decreased in some cats receiving intravenous $NaHCO_3^-$. Possible mechanisms include decreased Ca^{2+} resorption in proximal tubules because of Ca^{2+} complexing with excess HCO_3^-, alkalemia resulting in decreased [fCa^{2+}], and increased renal Na^+ excretion causing concurrent hypercalciuria.
3. Furosemide treatment: Furosemide directly inhibits Na^+ and Cl^- resorption in the ascending limb of the loop of Henle, and thus secondarily inhibits Ca^{2+} resorption in the ascending limb because the passive paracellular resorption of Ca^{2+} depends on charge gradients established by the Na^+-K^+-$2Cl^-$ cotransporter and associated K^+

Figure 11.6. *continued*

Compensations and progression that lead to secondary renal hyperparathyroidism (blue; arrow numbers 9–11 correspond to the following steps)

9. A lower [fCa^{2+}]:
   a. Stimulates the parathyroid glands to produce and release PTH
   b. Increases renal 1α-hydroxylase activity, which causes more formation of $1,25(OH)_2D$ from 25OHD and a return of [$1,25(OH)_2D$] to WRI, but progressive loss of vitamin-D receptors in the parathyroid gland creates resistance to $1,25(OH)_2D$, and uremia-related decreases in parathyroid gland expression of Klotho and the FGF23 receptor decrease FGF23-mediated parathyroid gland suppression, so PTH is secreted even with $1,25(OH)_2D$ being present[143]
   c. Leads to increased Ca^{2+} absorption in intestine and resorption from bone because of increased PTH and $1,25(OH)_2D$ production, and eventually return of [fCa^{2+}] to WRI
10. The ↑ [FGF23] and compensatory ↑ [PTH] promote less PO_4 resorption by the remaining tubules, and ↑ [FGF23] decreases intestinal PO_4 absorption via vitamin D–regulated Na^+-PO_4 cotransporters, all leading to a return of [Pi] to WRI.
11. The increased PTH activity may promote increased formation of FGF23 and thus further promote phosphaturia.

Maintenance of [fCa^{2+}] (thus [tCa^{2+}]), [PO_4], and [$1,25(OH)_2D$] within respective RIs despite fewer functional nephrons depends on ↑ [PTH], ↑ [FGF23], and responses to these mediators. Increased PTH formation occurs because of parathyroid gland hyperplasia and thus a state of secondary renal hyperparathyroidism. With further progression of renal disease, GFR decreases more and Klotho expression becomes markedly decreased; hyperphosphatemia develops because renal [PO_4] excretion is impaired. Hypocalcemia may also develop.

diffusion back into the tubular lumen.[176,177] With decreased transporter activity, the lumen has a more negative charge so more Ca^{2+} is retained in the lumen. Thus, when furosemide is used to promote diuresis, free hypocalcemia can develop. This calciuric effect of furosemide is used therapeutically to treat hypercalcemia. Thiazide diuretics should not be used in such hypercalcemia cases because they promote Ca^{2+} resorption.

D. Ca^{2+} binding with diffusible anions
   1. Ca^{2+}-binding anticoagulants: Ca^{2+} binds with EDTA, oxalate, or citrates given systemically or present in blood collection systems and transfused products. In one study in which dogs were massively transfused with either a volume of citrate-anticoagulated blood product equal to their estimated blood volume, over 24 h, or a volume > 50 % of their estimated blood volume, in 3 h, the average decrease in [fCa^{2+}] was near 0.3 mmol/L (1.2 mg/dL).[178] Hypocalcemia related to systemic increases in [citrate] may occur with plasmapheresis[179] or plateletpheresis.[180] EDTA-bound Ca^{2+} is not detected by routine assays for [tCa^{2+}], so [tCa^{2+}] decreases as well as [fCa^{2+}] with exposure to EDTA.
   2. Tetracycline is a Ca^{2+}-binding agent, and its rapid intravenous infusion may lower the [fCa^{2+}], but the [tCa^{2+}] remains WRI.[169]

E. Ca^{2+} deposition during fracture healing: Serum [tCa^{2+}] decreased slightly (about 0.6 mg/dL) during the early stages of fracture healing in dogs.[181] The decrease may not be enough to cause hypocalcemia by itself, but the Ca^{2+} deposition during fracture healing could contribute to hypocalcemia. [fCa^{2+}] may also decrease, but its assessment was not reported.

F. Other or unknown mechanisms
   1. Endotoxemia, sepsis, and other critical illness
      a. Hypocalcemia with decreased [fCa^{2+}] and variable [tCa^{2+}] is common in critically ill human patients without a primary endocrine disorder of Ca^{2+} homeostasis, particularly in septic patients, but also in nonseptic (e.g., trauma) patients.[182] Clinical signs usually associated with hypocalcemia are typically absent in this population. Similarly, hypocalcemia (sometimes only decreased [fCa^{2+}]) is common in septic and nonseptic critically ill dogs, cats, and horses.[183–186] Although explanations exist for hypocalcemia in some of the commonly listed critical conditions associated with hypocalcemia, the state of being critically ill appears to alter Ca^{2+} homeostasis and further promote hypocalcemia through relatively nonspecific mediators such as inflammatory cytokines (e.g., IL-1β and IL-6). Such cytokines can cause CaSR upregulation on parathyroid and renal tubular epithelial cells, thus decreasing PTH secretion, vitamin D activation, and tubular Ca^{2+} resorption, each of which promotes hypocalcemia.[187]
      b. Veterinary and human septic patients are commonly hypocalcemic with decreased [fCa^{2+}],[182,184–186] and experimental endotoxin administration to a variety of species (horses, dogs, pigs, sheep) causes a rapid decrease in [fCa^{2+}] and often [tCa^{2+}].[186,188,189] Other laboratory findings in dogs with experimental endotoxemia include a rapid decrease in [25OHD] and increase in [PTH].[183] The [PTH] was variable in a study of horses with experimental endotoxemia that induced milder decreases in [fCa^{2+}],[186] but critically ill foals with hypocalcemia appear to have increased [PTH] and evidence of bone resorption.[190] Potential contributors to sepsis- and endotoxin-induced hypocalcemia include extravasation of Ca^{2+} from blood due to increased vascular permeability, cytokine-mediated intracellular Ca^{2+} accumulation, hyporesponsive parathyroid

glands due to cytokines or hypomagnesemia (which is common with endotoxemia), and decreased Ca^{2+} mobilization because of decreased [25OHD] or inhibited activity of 1α-hydroxylase.[191] Vitamin D may play a different role in horses compared to other species given differences in renal 1α-hydroxylase and vitamin D metabolism in horses.

c. Colicky horses with a variety of gastrointestinal disorders frequently are hypocalcemic; the [fCa^{2+}] is decreased more frequently than the [tCa^{2+}].[192,193] Endotoxemia or sepsis may be involved in many cases.

2. Acute pancreatitis (dogs and cats)
   a. Hypocalcemia may be present in acute edematous or hemorrhagic pancreatitis in dogs. Hypocalcemia is common in cats with acute pancreatitis, with a decreased [fCa^{2+}] being more common than a decreased [tCa^{2+}]; free hypocalcemia was associated with a poorer prognosis.[194]
   b. The pathogeneses of the hypocalcemia and free hypocalcemia are not clearly identified and may involve multiple mechanisms.
      (1) The hypocalcemia is commonly attributed to the binding of Ca^{2+} to anions of fatty acids in the peritoneal cavity (forming Ca^{2+} soaps), fatty acids having been liberated from necrotic peritoneal fat because of actions of pancreatic lipase. However, there is little evidence to support the soap theory, and there is evidence to refute it.[195,196]
      (2) Other theories with variable support include abnormal hormonal regulation of the [fCa^{2+}] (involving glucagon, calcitonin, or PTH), endotoxemia, entry of Ca^{2+} into cells because of damaged membranes, and extravasation of protein-bound Ca^{2+} because of increased vascular permeability.[195–198]
3. Urinary tract obstruction:[199] Mild to marked hypocalcemia is commonly present; the [fCa^{2+}] is decreased more than the [tCa^{2+}]. The pathogenesis of this hypocalcemia is not clear, but increased plasma [Pi] and the resultant binding of fCa^{2+} to PO_4 may contribute. Roles for vitamin D and PTH have not been explored. Renal tubular damage occurring during obstruction may increase the renal excretion of Ca^{2+} after the obstruction has been cleared.
4. PO_4 enemas
   a. In reported feline cases, authors proposed that the hypocalcemia developed because of hyperphosphatemia.[200]
   b. Hyperphosphatemia may produce hypocalcemia through three processes:
      (1) Acutely, the high PO_4 load drives the following equation to the right in plasma ($Ca^{2+} + PO_4 \rightarrow Ca^{2+}$-$PO_4$ compounds), thus lowering the [fCa^{2+}] as colloidal particles form and are rapidly removed by macrophages.[27] The colloidal particles may represent calciprotein particles, i.e., aggregates of calcium-phosphate crystal–laden fetuin-A molecules.[201] There also may be more Ca^{2+}-PO_4 complexes in tissues.
      (2) Increased renal excretion of anionic PO_4 may obligate increased excretion of cations, including Ca^{2+}.
      (3) PO_4 inhibits the renal formation of $1,25(OH)_2D$.
5. Diabetic ketoacidosis: Many dogs and cats with diabetic ketoacidosis have unexplained hypocalcemia ([fCa^{2+}] and [tCa^{2+}]) at presentation to tertiary hospitals.[202–204] Concurrent disorders (pancreatitis, hyperadrenocorticism, sepsis, renal disease) or treatments may cause or contribute to the hypocalcemia. Persistent ketonuria would obligate renal loss of cations, including Ca^{2+}.

6. Blister beetle poisoning: Horses with blister beetle poisoning (cantharidiasis) are typically hypocalcemic.[205,206] Hypocalcemia has also been reported in a cantharidin-intoxicated alpaca in which decreased [fCa^{2+}] was documented concurrently with a low [PTH].[207] Blister beetles produce cantharidin, a toxicant that interferes with mitochondrial respiration and protein dephosphorylation via phosphatases. The pathogenesis of hypocalcemia is not understood, but in vitro studies suggest that cantharidin may inhibit osteoclastogenesis and osteoclast activation, thus decreasing osteolysis, and some phosphatase inhibitors impair PTH production.[207,208] Hypomagnesemia may be present and could contribute to hypocalcemia via a decreased PTH response. Loss of protein-bound Ca^{2+} into the intestinal tract may contribute to the decreased [tCa^{2+}] (but not to the decreased [fCa^{2+}]).
7. Myopathies: Hypocalcemia occurs in a variety of equine myopathies (e.g., transport tetany, exertional rhabdomyolysis, endurance-type exercise, monensin toxicosis, postanesthetic myositis, exhausted horse syndrome, atypical myoglobinuria, malignant hyperthermia, and selenium myopathy).[169,209–212] The pathogenesis of the hypocalcemia may be multifactorial (e.g., decreased intake, increased renal loss, increased movement of Ca^{2+} into damaged cells, and profuse sweating). Sweating in horses can create an alkalemia (hypochloremic metabolic alkalosis) that may increase Ca^{2+} binding to albumin and thus mildly lower the [fCa^{2+}]. Also, there may be direct loss of Ca^{2+} in sweat.[213]
8. Acute tumor lysis syndrome: PO_4 released from lysed cells may bind free Ca^{2+}, and the Ca^{2+}-PO_4 complexes may deposit in tissues or macrophages may engulf them as part of colloidal particles. PO_4 and lactate (produced by hypoxic neoplastic cells) may bind Ca^{2+} in renal tubular fluid and thus inhibit renal tubular resorption of Ca^{2+}.[214]
9. Grain overload (carbohydrate overload, acute carbohydrate ruminal engorgement, ruminal acidosis): Ruminants and camelids that ingest large amounts of fermentable carbohydrate may develop acute lactic acidosis, hypovolemia, azotemia, hyperphosphatemia, and hypocalcemia.[215] The pathogenesis of the hypocalcemia is not established but may relate to increased renal Ca^{2+} excretion, because Ca^{2+} may be bound to PO_4 or lactate in the tubular fluid. It may also relate to tissue deposition of Ca^{2+}-PO_4 complexes or their removal within colloidal particles engulfed by macrophages.
10. A minority of hyperthyroid cats have a decreased [fCa^{2+}] but [tCa^{2+}] WRI. The pathogenesis of the free hypocalcemia is not understood. The cats do have responsive secondary hyperparathyroidism.[216]
11. Horses after a cross-country race had free hypocalcemia but [tCa^{2+}] WRI.[217] In another study involving endurance rides (80 km), the average decrease in plasma [fCa^{2+}] was near 0.8 mg/dL; concurrently, the concentrations of lactate, Pi, and albumin increased. Data for [tCa^{2+}] were not reported.[218] The lower [fCa^{2+}] may have been due to several factors, including increased binding to albumin, increased binding to lactate, movement of Ca^{2+} from ECF to ICF, or loss of Ca^{2+} in sweat.
12. Hyperadrenocorticism in dogs: Mild hypocalcemia and/or free hypocalcemia have been reported in a small percentage of dogs with hyperadrenocorticism,[219] but hypocalcemia was not detected in a study of vitamin D status in 12 dogs with pituitary-dependent hyperadrenocorticism.[220] Although some evidence suggests that glucocorticoids do not have a direct, acute effect on calcium homeostasis in dogs,[221] dogs with spontaneous hyperadrenocorticism had hypercalciuria and increased [PTH] (secondary adrenal hyperparathyroidism) with unexplained increases in [Pi] compared to healthy dogs.[222] Free hypocalcemia was present in few of these dogs.

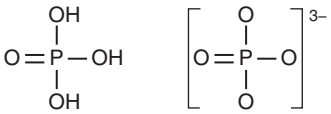
INORGANIC PHOSPHORUS (Pi) CONCENTRATION

I. Physiologic processes

A. The element phosphorus (not the adjective "phosphorous") is distributed throughout the body in the form of organic and inorganic phosphates. Organic phosphates (those containing carbon atoms) include phospholipids (e.g., membrane lipid bilayers), nucleic acids, and adenosine triphosphate (ATP), whereas bone, teeth, and cartilage are rich in inorganic PO_4. Most intracellular phosphates are largely immobilized.

B. Plasma phosphorus is present within organic and inorganic phosphates. Organic phosphates predominate, but clinical assays measure mostly the concentration of inorganic phosphates, and the results are reported in terms of the phosphorus in those inorganic phosphates, i.e., the inorganic phosphorus concentration denoted herein as [Pi].

C. Pi exists in different forms, depending on pH: $H_3PO_4 \leftrightarrow H^+ + H_2PO_4^- \leftrightarrow 2\,H^+ + HPO_4^{2-} \leftrightarrow 3\,H^+ + PO_4^{3-}$; these PO_4 molecules are called phosphoric acid, dihydrogen phosphate, hydrogen phosphate, and phosphate, respectively. At a pH of 7.4, predominant forms are $H_2PO_4^-$ and HPO_4^{2-} in a 1:4 ratio. Unless stated otherwise, all forms in this chapter are discussed inclusively as PO_4. About 10 % of PO_4 is reversibly bound by charge to cationic proteins, 35 % is reversibly bound by charge to nonprotein cations, and 55 % is free.[1,86]



Phosphoric acid Phosphate

D. Major factors that determine serum [Pi] (Figure 11.7)

1. Renal clearance of PO_4
   a. A major route of PO_4 excretion is via kidneys in most mammals, so disorders that cause decreased GFR tend to increase the serum [Pi]. Most PO_4 in the renal filtrate is resorbed in the proximal tubules via Na^+-PO_4 cotransporters.
   b. PTH is a potent phosphaturic agent. It binds to tubular epithelial cell receptors and causes internalization and destruction of Na^+-PO_4 cotransporters within minutes to days, depending on the transporter subtype, so less of filtered PO_4 is resorbed.
   c. FGF23, a small glycoprotein (a phosphatonin) produced by osteocytes and osteoblasts, also promotes PO_4 excretion. It reduces PO_4 resorption by reducing tubular expression of Na^+-PO_4 cotransporters.[86] FGF23 also influences plasma [PO_4] by downregulating 1α-hydroxylase, thus reducing synthesis of $1,25(OH)_2D$.[223] Greater [$1,25(OH)_2D$] promotes FGF23 production, as does greater plasma [PO_4].[85,86]
2. Absorption of PO_4 in the intestine: If animals are eating, they typically consume large quantities of PO_4, and in ruminants, much of the PO_4 entering the ruminant forestomach is from saliva; this PO_4 is important for local buffering and microbial growth. Most alimentary PO_4 is absorbed in the intestine of most species; some is absorbed by the forestomachs of ruminants, and the large intestine appears to be the major site for absorption in horses.[224,225] Absorption may occur paracellularly by passive diffusion, or transcellularly via Na^+-PO_4 cotransporters. Absorption is promoted by low dietary PO_4 and regulated in at least some species by vitamin D and FGF23.[86,226]
3. Resorption from bone: PTH stimulation of osteocytes and osteoblasts causes these cells to release factors that activate osteoclasts, and the resulting osteolysis releases PO_4 from bone. Because of the potent phosphaturic action of PTH, the release of PO_4 does not increase the [Pi] as long as renal function is adequate.

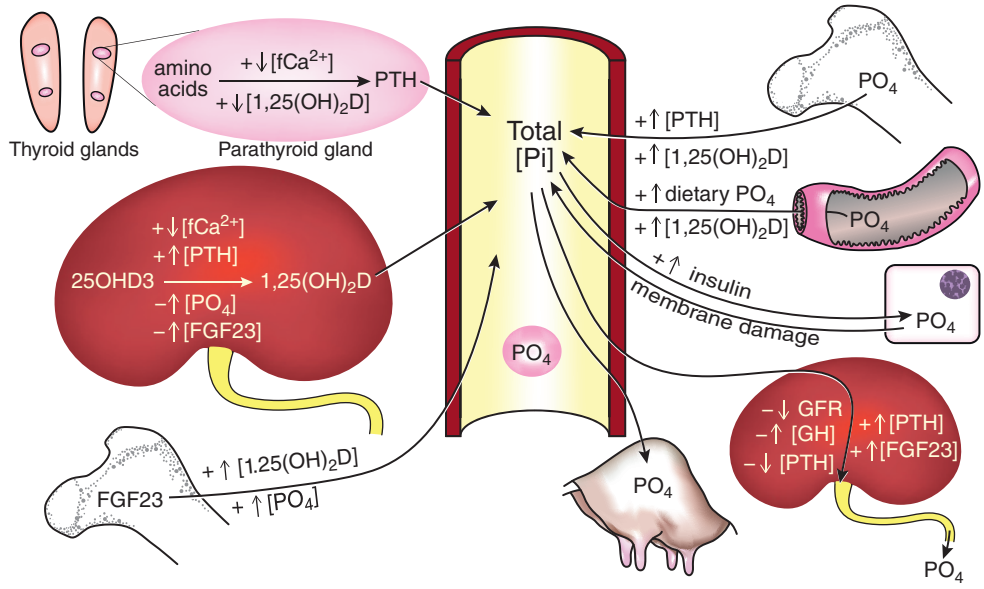


Figure 11.7. PO_4 kinetics and the production of PTH and $1,25(OH)_2D$. (Note: Horse kidneys lack 1α-hydroxylase and thus do not form $1,25(OH)_2D$.)

- PTH production in parathyroid glands is stimulated by ↓ $[fCa^{2+}]$ and ↓ $[1,25(OH)_2D]$ and inhibited by ↑ $[fCa^{2+}]$ and ↑ $[1,25(OH)_2D]$.
- Conversion of 25OHD to $1,25(OH)_2D$ in kidneys is catalyzed by 1α-hydroxylase. The activity of 1α-hydroxylase is promoted by ↓ $[fCa^{2+}]$ and ↑ PTH; it is inhibited by ↑ $[PO_4]$ and ↑ [FGF23].
- FGF23 is produced primarily by osteocytes; its production is stimulated by $1,25(OH)_2D$ and ↑ $[PO_4]$.
- PO_4 mobilization from bone (*right side of figure*) is promoted by ↑ $[1,25(OH)_2D]$ and ↑ PTH. Less PO_4 mobilization occurs with ↓ $[1,25(OH)_2D]$ and ↓ PTH; ↑ FGF23 indirectly decreases PO_4 resorption by decreasing the $[1,25(OH)_2D]$.
- PO_4 absorption in the intestine is promoted by ↑ $[1,25(OH)_2D]$ and ↑ dietary PO_4. Less PO_4 absorption occurs with ↓ $[1,25(OH)_2D]$ and ↓ dietary PO_4; ↑ FGF23 indirectly decreases PO_4 absorption by decreasing the $[1,25(OH)_2D]$.
- Insulin promotes the uptake of PO_4 by cells. Cell damage allows PO_4 to escape from cells and enter plasma.
- Urinary excretion of PO_4 is enhanced by ↑ PTH and ↑ FGF23; excretion is reduced by ↓ GFR, ↓ PTH, and ↑ GH activity.
- During lactation, a large amount of PO_4 is excreted via milk.
- PO_4 present in plasma is mostly in two forms (HPO_4^{2-} and $H_2PO_4^-$), but the measured phosphorus is reported in terms of inorganic phosphorus (Pi).
- In vitro hemolysis or delayed removal of serum or plasma allows PO_4 in RBCs to enter serum or plasma and thus cause an erroneous [Pi].

+, positive effector (stimulates the process); –, negative effector (inhibits the process)

4. Shifting of PO_4 between ICF and ECF compartments: Intracellular biochemical processes that involve phosphorylation need PO_4 from ECF. Insulin promotes PO_4 entry into cells by enhancing the movement of glucose into cells. Once in cells, glucose is phosphorylated to enter glycolytic or glycogen-synthesis pathways. The binding of

free PO_4^{3-} to glucose or other organic molecules reduces the ICF concentration of free PO_4^{3-} and allows more PO_4 to enter cells from the ECF.

5. A large amount of PO_4 is excreted in milk during lactation.
6. Animal age
   a. GH is reported to be largely responsible for the increased [Pi] in young, growing animals. GH increases the renal tubular resorption of PO_4.[227]
   b. Serum [Pi] is typically greater in young, growing animals than in the adult animals used to establish most RIs.[9,11] In pups (< 12 wk), the serum [Pi] RIs were about 5.7–10.8 mg/dL, whereas the RIs for adult dogs were about 2.5–5.5 mg/dL. In kittens (4–6 wk to 20–24 wk), serum [Pi] RIs were about 5.0–10.0 mg/dL, whereas adult cat intervals were about 1.8–6.4 mg/dL. A corresponding age-related difference is that these young animals have greater serum alkaline phosphatase activity than mature animals (usually < 3 times the URL for adults).

II. Analytical concepts of [Pi]
   A. Sample
      1. Serum or heparinized plasma may be used.
      2. In vitro hemolysis or delayed removal of serum from a blood clot may increase serum or plasma [Pi] because PO_4 is liberated from RBCs.[29]
   B. Assays
      1. In most common assays, PO_4 reacts with ammonium molybdate to form a colored complex that is measured by photometry.
      2. In some systems, conjugated bilirubin may interfere with the assay to produce falsely decreased results (*pseudohypophosphatemia*),[228] whereas, in other assay systems, bilirubin has no effect or may produce falsely increased values (*pseudohyperphosphatemia*).
      3. In spectrophotometric assays that use the strongly acidic ammonium molybdate reagent, the reagent can precipitate certain immunoglobulins to produce a turbidity that results in a falsely increased [Pi] by 1–2 mmol/L (≅ 3–6 mg/dL).[229,230] This positive interference has been seen in samples with monoclonal gammopathies despite assay modifications such as the addition of detergent to prevent protein precipitation.
   C. Methods of expressing concentrations
      1. [Pi] is often referred to as the phosphorus concentration. More accurately, [Pi] is the serum PO_4 reported as phosphorus in mg/dL. It is not reported as a PO_4 concentration in mg/dL because the amount of each form of PO_4 changes with pH, and each form has a slightly different relative molecular mass. This problem is overcome by reporting PO_4 in SI units of mmol/L (the number of molecules per volume rather than mass of molecules per volume).
      2. Relationship between [PO_4] and [Pi]
         a. 1 mmol of $H_2PO_4^-$ weighs 97 mg. In 1 mmol of $H_2PO_4^-$ there are 31 mg of P. Therefore, a solution of 97 mg/dL of $H_2PO_4^-$ contains 31 mg/dL of P.
         b. 1 mmol of HPO_4^{2-} weighs 96 mg. In 1 mmol of HPO_4^{2-} there are 31 mg of P. Therefore, a solution of 96 mg/dL of HPO_4^{2-} contains 31 mg/dL of P.
         c. Because of the equilibrium among the different PO_4 molecules at a pH of 7.4, 1 mmol of PO_4 averages about 96.2 mg/dL.
   D. Unit conversion for [Pi]: mg/dL × 0.3229 = mmol/L (SI unit)[31]

Table 11.4. Disorders and conditions that cause hyperphosphatemia
Decreased urinary PO_4 excretion
Disorders that decrease GFR (see prerenal, renal, and postrenal azotemias in Chapter 8)*
Urinary bladder rupture or urine leakage into tissues
Decreased [PTH] or activity (primary hypoparathyroidism or pseudohypoparathyroidism)
Acromegaly
Increased PO_4 absorption from the intestine
PO_4 enema, ingestion of PO_4 urinary acidifier, oral PO_4 salts (cattle)
Increased vitamin D (Tables 11.2, 11.10, 11.12)
Ischemic intestinal lesions (maybe also shift from ICF to ECF)
Diet with a low Ca^{2+}:PO_4 ratio (rare)
Shift of PO_4 from ICF to ECF
Myopathies: endurance rides in horses, exertional rhabdomyolysis, malignant hyperthermia
Acute tumor lysis syndrome
Other or unknown mechanisms
Hyperthyroidism in cats
Lactic acidosis
Hyperadrenocorticism in dogs
Pseudohyperphosphatemia
In vitro hemolysis or delayed removal of serum or plasma from blood samples
Hyperbilirubinemia or monoclonal gammopathies (analytical effect; assay dependent)
Contamination of sample with PO_4-containing alteplase or heparinized saline

* A relatively common disorder, condition, or mechanism
Note: The [Pi] in growing mammals may be up to about 3 mg/dL greater than the URL for [Pi] in adults of the species.

III. Hyperphosphatemia (Table 11.4)
  A. Decreased urinary PO_4 excretion
    1. Disorders that decrease GFR (see prerenal, renal, and postrenal azotemias in Chapter 8): Hyperphosphatemia occurs because PO_4 is not adequately removed from plasma. The magnitude tends to parallel the severity of azotemia in dogs, cats, and cattle, but it may not in horses, some of which are hypophosphatemic when in renal failure.
    2. Urinary bladder rupture or leakage of urine into tissues: Hyperphosphatemia results from decreased urinary excretion of PO_4 from the body, and from movement of peritoneal or tissue PO_4 to blood.
    3. Decreased [PTH] or activity (primary hypoparathyroidism or pseudohypoparathyroidism) (see Total and Free Calcium (tCa^{2+}, fCa^{2+}) Concentrations, Section VI.B).
    4. Acromegaly: GH increases tubular PO_4 resorption.[227]
  B. Increased PO_4 absorption from the intestine
    1. PO_4 enemas,[231,232] ingestion of a PO_4 urinary acidifier,[233] or, potentially, oral administration of PO_4 salts to cattle[234]
    2. Increased vitamin D
      a. Hypervitaminosis D in ruminants: perhaps increased intestinal absorption of PO_4 or increased bone resorption

b. Cholecalciferol intoxication in dogs (Total and Free Calcium (tCa^{2+}, fCa^{2+}) Concentrations, Section V.A.2)

3. Ischemic intestinal lesions: Devitalized intestinal mucosa allows PO_4 to enter plasma (and peritoneal fluid) from the intestinal lumen. Also, shifting of PO_4 from ICF to ECF may be involved.[235]
4. Diets with low Ca^{2+}:PO_4 ratio (rare)

C. Shift of PO_4 from ICF to ECF
1. Myopathies (endurance rides in horses, exertional rhabdomyolysis, and malignant hyperthermia): release of PO_4 from damaged myocytes[210–212,236]
2. Acute tumor lysis syndrome: release of PO_4 from necrotic neoplastic cells[214]

D. Other or unknown mechanisms
1. Hyperthyroidism in cats: Thyroxine may promote osteoclastic activity to cause release of Ca^{2+} and PO_4 from bone. Hyperthyroid cats may have hyperphosphatemia, a decreased [fCa^{2+}], and an increased [PTH].[237] The reason for the decreased [fCa^{2+}] has not been established but has been considered the stimulus for the increased [PTH].
2. Lactic acidosis:[238] This causes hyperphosphatemia more frequently than other metabolic acidoses, but the mechanisms have not been completely explained. The hyperphosphatemia might be linked to the acidemia mechanism, i.e., anaerobic glycolysis causes a net degradation of adenosine triphosphate (ATP) and thus increases cellular production of H^+ and PO_4 (Figure 9.11).[239] The [Pi] in human patients with hyperphosphatemia attributed to lactic acidosis has been as much as or, rarely, more than 2 times the URL.[240,241]
3. Hyperadrenocorticism in dogs: The serum [Pi] in 68 dogs with hyperadrenocorticism was significantly greater than in 20 hospitalized dogs without clinical signs of hyperadrenocorticism. The dogs with hyperadrenocorticism also had significantly greater plasma [PTH], but no significant differences for [tCa^{2+}] or [fCa^{2+}] were found between the groups.[219]

E. Pseudohyperphosphatemia
1. In vitro hemolysis or delayed removal of serum or plasma from blood may create a higher [PO_4] as PO_4 from RBCs enters the fluid.
2. Falsely increased [Pi] has been described in samples with hyperbilirubinemia or a monoclonal gammopathy (see analytical section above).
3. Contamination of a blood sample with alteplase or heparinized saline
   a. Alteplase is a plasminogen activator inhibitor that contains phosphoric acid to create an acidic pH.[242]
   b. Some heparin solutions used to maintain patency of indwelling catheters contain sufficient [PO_4] to greatly increase a blood sample's [Pi] if the sample is drawn from an indwelling line without discarding the appropriate volume of fluid prior to collecting the sample, or if too much of the heparin is used to heparinize a syringe.[243]

IV. Hypophosphatemia (Table 11.5)

A. Increased urinary PO_4 excretion
1. Prolonged diuresis: Less filtered PO_4 is resorbed in various polyuric states when increased flow through the tubules diminishes time for PO_4 resorption.
2. Increased PTH or PTHrP activity (Tables 11.2 and 11.8): As described above, PTH and PTHrP are potent phosphaturic agents that reduce tubular resorption of PO_4.
3. Fanconi syndrome: Animals with *Fanconi syndrome* have a proximal tubular defect that decreases tubular resorption of glucose, amino acids, and PO_4. Some people restrict the

Table 11.5. Disorders and conditions that cause hypophosphatemia
Increased urinary PO_4 excretion
Prolonged diuresis
Increased PTH or PTHrP activity (Tables 11.2 and 11.8)
Fanconi syndrome
Endotoxemia and sepsis
Paraneoplastic hypophosphatemia (FGF23)
Decreased intestinal PO_4 absorption
Prolonged anorexia or PO_4-deficient diet*
PO_4-binding agents
Hypovitaminosis D
Intestinal malabsorption
Shift of PO_4 from ECF to ICF
Hyperinsulinism (endogenous or exogenous)
Refeeding syndrome
Glucose infusion
Alkalemia due to respiratory alkalosis or metabolic alkalosis
Generalized seizures (likely from PO_4 shifts)
Defective mobilization of PO_4 from bone
Postparturient paresis (milk fever) and eclampsia in bitches
Bisphosphonates (e.g., pamidronate, alendronate)
Other or unknown mechanisms
Equine renal disease (failure)*
Halothane anesthesia in horses
Pseudohypophosphatemia
Hyperbilirubinemia (analytical effect; assay dependent)
Extreme neoplastic leukocytosis (in vitro effect)

* A relatively common disorder, condition, or mechanism

term *Fanconi syndrome* to inherited or congenital forms, whereas others include acquired tubular nephropathies such as those related to copper-associated hepatopathy or ingestion of certain jerky treats. The decreased resorption of PO_4 in dogs with Fanconi syndrome may cause hypophosphatemia and phosphaturia in some dogs[244,245] but not others.[246,247]

4. Endotoxemia and sepsis: Hypophosphatemia is recognized as a manifestation of endotoxemia in horses[186] and people. The hypophosphatemia is at least partially due to increased renal excretion of PO_4 because of increased PTH activity, but inflammatory cytokines (IL-6 and TNF-α)[248] and alkalemia (see shifts in next section) may also play roles. Other mechanisms related to patients' specific disorders or treatment may also contribute.
5. Paraneoplastic hypophosphatemia: Tumor-induced osteomalacia is associated with several neoplasms in people and is characterized by renal PO_4 loss and hypophosphatemia. Benign or malignant neoplastic cells produce phosphatonins, most commonly FGF23, which promotes phosphaturia and inhibits 1α-hydroxylase, thus impairing $1,25(OH)_2D$ formation.[249,250]

B. Decreased intestinal PO_4 absorption
   1. Prolonged anorexia or a PO_4-deficient diet: less dietary PO_4 available for absorption
   2. PO_4-binding agents: interference with PO_4 absorption
   3. Hypovitaminosis D: decrease in a promoter of PO_4 absorption
   4. Intestinal malabsorption: decreased PO_4 absorption because of decreased functional mucosal tissue

C. Shift of PO_4 from ECF to ICF
   1. Hyperinsulinism (endogenous or exogenous)
      a. Insulin promotes entry of PO_4 into cells to phosphorylate the glucose that enters the cells. High insulin activity could result from administration of insulin or an insulin analog (e.g., insulin lispro), an insulin-secreting neoplasm, or in response to glucose infusion.
      b. Xylitol toxicity: Dogs are sensitive to xylitol, a xylose-derived 5-carbon sugar alcohol used mostly as an artificial sweetener. Ingestion causes insulin secretion with subsequent cellular uptake of glucose, PO_4, and K^+ that can cause hypoglycemia, hypophosphatemia, and hypokalemia.[251,252]
   2. Refeeding syndrome, the metabolic disturbances caused by reinstitution of nourishment to starved or severely malnourished patients, is characterized by hypophosphatemia, hypomagnesemia, and hypokalemia. It has been recognized in cats with hepatic lipidosis, marked starvation, cholangiohepatitis, and malignant neoplasms.[253–255] The hypophosphatemia is probably due to an acute shift of PO_4 into cells because of insulin activity, and to increased cellular metabolism combined with a preexisting PO_4-depleted state.[86]
   3. Glucose infusion (in horses) led to hypophosphatemia.[256] The hypophosphatemia may be due to increased renal excretion of PO_4 secondary to solute diuresis and also to insulin-induced movement of PO_4 from ECF to ICF secondary to glucose uptake by cells.
   4. Alkalemia due to respiratory alkalosis or possibly metabolic alkalosis
      a. Experimentally-induced respiratory alkalosis caused hypophosphatemia and an increase in muscle glycolysis;[257] experimentally-induced metabolic alkalosis also created hypophosphatemia, but to a lesser extent.[258] Hypophosphatemia due to hyperventilation may be more common than is recognized.[259]
      b. Phosphofructokinase is the rate-limiting enzyme in glycolysis, and alkalemia stimulates its activity. Thus, alkalemia promotes PO_4 movement into RBCs and other cells (e.g., myocytes) because of the increased phosphorylation during accelerated glycolysis.[257,259]
   5. Generalized seizures may be followed by hypophosphatemia that resolves within a few hours.[260] Intracellular shifting of PO_4 is likely, but the specific mechanism has not been clarified.

D. Defective mobilization of PO_4 from bone
   1. Postparturient paresis (milk fever): Cows cannot mobilize sufficient Ca^{2+} and PO_4 from bones to replace the Ca^{2+} and PO_4 that are lost in milk.
   2. Eclampsia in bitches: the same mechanism as for milk fever
   3. Pamidronate and alendronate, bisphosphonates that inhibit bone resorption, promote hypophosphatemia and hypocalcemia; hypophosphatemia may be severe.[171,261]

E. Other or unknown mechanisms
   1. Equine renal disease (failure): Hypophosphatemia may be seen in equine renal disease that causes concurrent hypercalcemia.[262] However, it is not a consistent finding, and the pathogenesis is not known.

2. Halothane anesthesia in horses:[263] After prolonged halothane anesthesia (12 h), six horses had mild hyperphosphatemia for the first hour, but then hypophosphatemia developed and persisted for 3–4 d. The pathogenesis of the hypophosphatemia was not determined.

F. Pseudohypophosphatemia
1. Hyperbilirubinemia: As noted in the analytical section (Inorganic Phosphorus (Pi) Concentration, Section II.B), the presence of conjugated hyperbilirubinemia can result in a falsely decreased [Pi] with certain assays.
2. Extreme neoplastic leukocytosis: There is most likely an in vitro shift of PO_4 into neoplastic cells because of their high metabolic rate; in a reported human case, placing a blood sample on ice prevented the pseudohypophosphatemia.[264]

TOTAL AND FREE MAGNESIUM (tMg^{2+}, fMg^{2+}) CONCENTRATIONS

Note: As with fCa^{2+} data, several factors make it difficult to interpret reported fMg^{2+} data in some publications. The following should be considered whenever one is interpreting or communicating [fMg^{2+}] results.

1. *Was sample collection and handling adequate to ensure accurate results? What steps were taken to prevent inaccuracies caused by excess heparin or exposure to air?*
2. *Was the reported [fMg^{2+}] a measured concentration or a normalized concentration?*
3. *Are the terms hypermagnesemia and hypomagnesemia referring to [fMg^{2+}] or [tMg^{2+}]?*

I. Physiologic processes

A. Serum or plasma Mg^{2+} is distributed into three major fractions. All Mg^{2+} in body fluids is ionized, but some is free, and some is bound to anionic molecules.
1. *Free magnesium* (*fMg^{2+}*): About 55 % of tMg^{2+} is fMg^{2+} present as free ions in plasma H_2O. The subset of fMg^{2+} not neutralized by electrostatic interactions with other ions is the portion of tMg^{2+} that is hormonally regulated and contributes to pathologic states.
2. Bound Mg^{2+}
   a. About 30 % of tMg^{2+} is bound to negatively charged sites on proteins (albumin and globulins).
   b. About 15 % of tMg^{2+} is bound to negatively charged sites on NPA such as citrate and PO_4.
3. In a group of 28 clinically healthy horses, the [fMg^{2+}] averaged 58 % of the [tMg^{2+}], and 9 % was bound to NPA.[4] In another study, the [fMg^{2+}] averaged about 60–65 % of the [tMg^{2+}] in adult horses, with considerable variation around the mean.[15]
4. In 1- to 7-d-old foals, [tMg^{2+}] was about 10 % greater than adult values while [fMg^{2+}] did not differ between foals and adults.[15]
5. Often-used terminology might suggest that there are different types of Mg^{2+} interactions with anionic proteins and with NPA, because the former is often referred to as "protein-bound Mg^{2+}" and the latter as "anion-bound Mg^{2+}" or "complexed Mg^{2+}." However, the involved protein binding sites are anionic and both associations are reversible charge-based complexes.

B. Mg^{2+} is located in bones (about 50–60 % of it), in soft tissues, and in the ECF, including blood (1 %). Except in cattle, the [tMg^{2+}] in RBCs is greater than in plasma (or serum).[265] In general, the small amount of available fMg^{2+} in the ICF compartment of other tissues is at a similar concentration as the [fMg^{2+}] in ECF.

C. Major factors that determine the serum [tMg^{2+}]: Mg^{2+} homeostasis is maintained primarily by intestinal absorption and renal resorption of Mg^{2+}. No hormone with primary Mg^{2+}-regulatory functions has been identified.
   1. Hypoproteinemia decreases the amount of bound Mg^{2+} and thus may cause or contribute to hypomagnesemia (decreased [tMg^{2+}]), but it is changes in [fMg^{2+}] that are of physiologic and pathologic significance.
   2. Intestinal absorption[55]
      a. In ruminants, Mg^{2+} is absorbed by the rumen and reticulum (and the intestine in calves). There is evidence for two transcellular pathways linked to Na^+-K^+-adenosine triphosphatase (ATPase):[266] (i) uptake through a Mg^{2+} channel, likely formed by TRPM proteins and driven by potential difference across the apical membrane, and (ii) cotransport of Mg^{2+} with anions down concentration gradients of Mg^{2+} and the involved anions. Although the large intestine is not a significant site of Mg^{2+} absorption in healthy adult cattle, it is in calves prior to intake of solid food, and can be used as a therapeutic route for Mg^{2+} administration in adults.
      b. In monogastric animals, Mg^{2+} absorption occurs throughout the small intestine and colon and is enhanced by vitamin D and inhibited by high dietary Ca^{2+} or PO_4.[267] Intestinal absorption in people is described as occurring through paracellular and transcellular pathways, mostly paracellular ones, by passive movement down a concentration gradient.[268] Tight junctional complexes between enterocytes modulate absorption; mutations in genes for junctional proteins (e.g., claudins) that facilitate absorption can cause disease through altered absorption. Active transcellular Mg^{2+} absorption occurs mostly through specific ion channels (TRPM6); their expression or activity increases with increasing pH and decreases with decreasing pH (more acidic) or increased [fMg^{2}].
   3. Renal resorption or excretion
      a. Plasma Mg^{2+} not bound to proteins (about 70 % of tMg^{2+}) passes freely through the glomerular filtration barrier. Thus, a decreased GFR can reduce renal excretion of Mg^{2+} and cause hypermagnesemia.
      b. In health, nephrons recover 95–99 % of filtrate Mg^{2+} by both paracellular and transcellular (TRPM6) resorption as described for intestinal absorption. In contrast to other ions, the proximal tubule is not the major site of Mg^{2+} resorption, but some is resorbed from proximal tubules along a concentration gradient generated by Na^+ and H_2O resorption.
         (1) Paracellular resorption in the loop of Henle: Most (up to about 70 %) of the resorption occurs through epithelial cell tight junctions in the ascending limb of the loop of Henle down an electrochemical gradient established by the Na^+-K^+-$2Cl^-$ cotransporter. In hypercalcemic or hypermagnesemic states, binding of Ca^{2+} and perhaps Mg^{2+} to the CaSR on thick ascending epithelial cells inhibits this cotransporter, thus altering the transepithelial electrochemical gradient and decreasing paracellular Ca^{2+} and Mg^{2+} resorption (enhances Ca^{2+} and Mg^{2+} excretion).[269]
         (2) Transcellular resorption in the distal tubule: About 10–15 % of renal Mg^{2+} resorption occurs actively through TRPM6 (as in the colon) in the apical membrane of distal convoluted tubule epithelial cells.[269] Resorption is facilitated by uptake of luminal Na^+ and Cl^- by the thiazide-sensitive Na^+-Cl^- cotransporter. Hypomagnesemia increases Mg^{2+} (and Ca^{2+}) resorption by increasing expression of TRMP6; increased fMg^{2+} inhibits TRMP6 expression.

Increases in pH occurring in metabolic alkaloses promote expression of TRMP6 (increased Mg^{2+} resorption) whereas decreases in pH associated with metabolic acidoses reduce TRMP6 expression (increased Mg^{2+} excretion), likely via basolateral pH changes sensed by the transporting cells. Resorption through TRMP6 is also promoted by epidermal growth factor and estrogen.

c. If the amount of Mg^{2+} entering the tubules exceeds the nephron's tubular resorptive capacity, excess Mg^{2+} is excreted. Osmotic diuresis and diuresis caused by loop or thiazide diuretics increase renal excretion of Mg^{2+}. Thiazides also reduce TRMP6 expression, thus decreasing Mg^{2+} resorption.[270]

d. If Mg^{2+} is present in bovine urine, then the cow is not expected to be hypomagnesemic because the presence of Mg^{2+} typically indicates that the transport maximum for tubular resorption has been exceeded. Thus, finding Mg^{2+} in urine indicates that a cow does not have hypomagnesemic tetany (grass tetany) unless there is also a defect in the resorption of Mg^{2+}.

4. Mammary gland during lactation: The ratio of milk [tMg^{2+}] to serum [tMg^{2+}] in cows is about 5:1.
5. Mg^{2+} in feces may represent unabsorbed dietary Mg^{2+} but also loss of endogenous Mg^{2+}.

D. Hormonal factors affecting Mg^{2+} homeostasis

1. Antidiuretic hormone (arginine vasopressin), PTH, glucagon, calcitonin, and β-adrenergic agonists have all been shown to stimulate Mg^{2+} absorption in the cortical thick ascending limb of the loop of Henle, at least in vitro.
2. In cattle, PTH administration can increase serum [tMg^{2+}] acutely by increasing intestinal Mg^{2+} absorption and increasing Mg^{2+} resorption in renal tubules and bone.[271]
3. Administration of $1,25(OH)_2D3$ to cattle generally reduces plasma [tMg^{2+}], perhaps secondary to hypercalcemia which would decrease passive tubular resorption of Mg^{2+}. This effect is greater than the increase in gastrointestinal paracellular Mg^{2+} absorption induced by $1,25(OH)_2D3$.[271]
4. Thyroxine tends to decrease the plasma [tMg^{2+}] by increasing Mg^{2+} excretion in urine and feces.[272]
5. Aldosterone promotes increased fecal and urinary Mg^{2+} excretion. Experimentally, aldosterone infusions decreased ruminal Mg^{2+} absorption.[273] Decreased aldosterone activity increases serum [tMg^{2+}].

II. Analytical concepts of [tMg^{2+}]

A. Sample

1. Serum and heparinized plasma may be used. Serum [tMg^{2+}] was stable for 7 d at 9 °C and for 2 d at room temperature.[29] Serum [fMg^{2+}] is not stable at 4 °C but is at –20 °C.[4] [tMg^{2+}] was mildly decreased (< 9 %) in canine heparinized plasma stored at 20 °C for 1–3 d, and it was mildly increased (< 6 %) in canine heparinized plasma by day 3 of heparinized whole blood storage at 20 °C.[274]
2. Blood anticoagulants that substantially bind Mg^{2+} (e.g., EDTA, citrate, and oxalate) should not be used in samples for [tMg^{2+}] assays.
3. Except in cattle, in vitro hemolysis or delayed removal of serum from a blood clot increases serum [tMg^{2+}] because Mg^{2+} is liberated into the plasma or serum from the RBCs. In cattle, plasma and RBC concentrations of Mg^{2+} are similar.[265]
4. Postmortem aqueous humor and vitreous humor samples can be used to reflect the antemortem plasma [tMg^{2+}]. The [tMg^{2+}] was more stable in vitreous humor (up to 48 h) than in aqueous humor (up to 24 h).[275]

B. Clinical assays: Common assays are photometric and measure [tMg^{2+}] through use of metallochromic indicators or dyes that change colors when they selectively bind Mg^{2+}. Atomic absorption spectrometry is the reference method but is not used in most clinical laboratories.
C. Unit conversion: mg/dL × 0.4114 = mmol/L; mEq/L × 0.500 = mmol/L (SI unit)[31]

III. Analytical concepts of [fMg^{2+}]
A. Terms: Most of the statements in the fCa^{2+} section also apply to terms used for fMg^{2+} concentrations. For consistency, these abbreviations are used: magnesium (Mg^{2+}), free magnesium (fMg^{2+}), and total magnesium (tMg^{2+}). In clinical jargon and in many veterinary publications, fMg^{2+} is often called *ionized magnesium* and is abbreviated as iMg, iMg^{2+}, or just Mg^{2+}, and thus there is a potential for miscommunication. All the measured serum or plasma Mg^{2+} is ionized (charged), whether free or complexed to protein and NPA by charge associations.
B. Sample: Most statements for [fCa^{2+}] also apply to [fMg^{2+}] because fCa^{2+} and fMg^{2+} are both divalent cations that bind to anions (see Total and Free Calcium (tCa^{2+}, fCa^{2+}) Concentrations, Section II.A). Whole blood without an anticoagulant may be used in some point-of-care analyzers. Heparin is a polyanion that complexes fMg^{2+}, perhaps even more so than fCa^{2+}, especially with excessive amounts of heparin.[276]
C. Clinical assays for [fMg^{2+}] are generally restricted to blood gas analyzers that measure [fMg^{2+}] by potentiometry using Mg^{2+}-selective electrodes. Because Ca^{2+} ions interfere with Mg-selective electrodes, [fCa^{2+}] must be measured concurrently and the result used to correct for interference prior to the analyzer generating a result. Zinc ions also interfere, so zinc-balanced heparin syringes should not be used.
D. If the analyzer used for measuring [fMg^{2+}] contains a pH electrode, a *normalized [fMg^{2+}]* may be calculated and reported to show the expected [fMg^{2+}] if the pH of the sample were 7.4.
1. The effect of pH on Mg^{2+} shifts is about half that of Ca^{2+}: a 2–3 % decrease in [fMg^{2+}] for each 0.1 increase in pH, and vice versa.
2. Some laboratories receiving serum samples through the mail routinely acidify the samples to bring the pH into the analytical range of the analyzer, and then apply an empirical formula to adjust the [fMg^{2+}] to what it would be at a pH of 7.4. These pH-normalized values do not provide the actual [fMg^{2+}] in the patient. Acid-adjustment of samples with a pH > 7.8 may yield falsely decreased results because of irreversible Mg^{2+} reactions.[277]
E. Unit conversion: mg/dL × 0.4114 = mmol/L; mEq/L × 0.5 = mmol/L (SI unit)[31]

IV. Hypermagnesemia (↑ [tMg^{2+}]) and free hypermagnesemia (↑ [fMg^{2+}]) (Table 11.6): Increased [tMg^{2+}] may occur with disorders of fMg^{2+} homeostasis or with disorders that increase plasma concentrations of fMg^{2+}-binding anions.
A. Decreased urinary excretion: This occurs with renal failure and other causes of decreased GFR; in chronic renal failure, decreased GFR typically causes hypermagnesemia and free hypermagnesemia despite decreased tubular resorption of Mg^{2+}.[278]
B. Shift of Mg^{2+} from ICF to ECF: Except in cattle, in vitro hemolysis, active in vivo hemolysis, or the delayed removal of serum from a blood clot allows RBC Mg^{2+} to be added to the serum concentrations of fMg^{2+} and tMg^{2+} (pseudohypermagnesemia when occurring in vitro).
C. Increased intestinal absorption of Mg^{2+}: This occurs with excess oral administration of MgO (magnesium oxide) and $Mg(OH)_2$ (magnesium hydroxide) to cattle[279] or with excess oral administration of $MgSO_4$ (magnesium sulfate, Epsom salt) to horses.[280] [fMg^{2+}] and [tMg^{2+}] may be increased.

Table 11.6. Disorders or conditions that cause hypermagnesemia and free hypermagnesemia[a]
Decreased urinary excretion
Renal failure and other causes of decreased GFR*
Shift of fMg^{2+} from ICF to ECF: active in vivo hemolysis (not cattle)
Increased intestinal absorption of Mg^{2+}
MgO, $Mg(OH)_2$, or similar antacids or cathartics (cattle)
$MgSO_4$ (horses)
Other or unknown mechanisms
Associated with colic
Postpartum in cattle
Excess intravenous infusion of Mg^{2+}
Perianal gland carcinomas (dogs)
Pseudohypermagnesemia
In vitro hemolysis or delayed removal of serum or plasma from blood samples (not cattle)

* A relatively common disorder, condition, or mechanism
[a] All the listed disorders may cause an ↑ [tMg^{2+}] and an ↑ [fMg^{2+}].

D. Other or unknown mechanisms
   1. Associated with colic: In horses, increased [fMg^{2+}] was common prior to and after colic surgery, but the reasons were not determined.[192]
   2. Postpartum in cattle: A transient increase in [tMg^{2+}] and [fMg^{2+}] occurs in cattle postpartum. The increase may be related to increased PTH activity causing increased renal resorption of Mg^{2+} and increased Mg^{2+} mobilization from bone.[281]
   3. Excess intravenous infusion of Mg^{2+}, including $MgSO_4$ to calm horses at equestrian events,[282] may increase [fMg^{2+}] and [tMg^{2+}] and cause hypermagnesemia that would be exacerbated by decreased GFR.
   4. Dogs with malignant epithelial perianal gland neoplasms had serum tMg^{2+} concentrations 2–3× those of dogs with benign perianal gland masses or of dogs that were surgical candidates without neoplastic disease ([tMg^{2+}] was measured by atomic absorption spectrophotometry). There was no assessment of Ca^{2+} or PTHrP status.[283]
   5. Addison's disease and hypothyroidism might cause hypermagnesemia.
   6. Lack of insulin in diabetes mellitus may contribute to free hypermagnesemia and perhaps increased [tMg^{2+}] by decreasing cellular uptake of Mg^{2+}.

E. Pseudohypermagnesemia (not in cattle) from in vitro hemolysis or delayed removal of serum or plasma from blood samples

V. Hypomagnesemia (↓ [tMg^{2+}]) and free hypomagnesemia (↓ [fMg^{2+}]) (Table 11.7): Decreased [tMg^{2+}] may be from disorders of fMg^{2+} homeostasis or from disorders that decrease fMg^{2+}-binding anions. Unless stated otherwise, the listed disorders can cause hypomagnesemia and free hypomagnesemia. Normomagnesemia does not exclude a decrease in total body Mg^{2+} content.

A. Hypoproteinemia: decreased Mg^{2+} bound to proteins (decreased [tMg^{2+}] without decreased [fMg^{2+}])

Table 11.7. Disorders and conditions that cause hypomagnesemia or free hypomagnesemia[a]

[tMg^{2+}]	[fMg^{2+}]	
↓	WRI	Hypoproteinemia*
↓	↓	Inadequate ruminal or intestinal absorption of Mg^{2+}
↓	↓	Prolonged anorexia or poor feed intake*
↓	↓	Calves on whole milk diet
↓	↓	Grass tetany in cattle*
↓	↓	Enteric disorders (chronic enteropathies, enteritis, enterocolitis, intestinal strangulation, intestinal resection, ileus, colic)
↓	↓	Excess urinary excretion of Mg^{2+}
↓	↓	Osmotic diuresis*
↓	↓	Ketonuria*
↓	↓	Fluid therapy
↓	↓	Diuretics
↓	↓	Other therapeutic agents affecting renal tubule function: aminoglycoside antibiotics, cisplatin, carboplatin, cyclosporine, amphotericin B, calcineurin inhibitors, and epidermal growth factor inhibitors
WRI[b]	↓	Mg^{2+} binding to anions (citrate, fatty acid anions)
		Other or unknown mechanisms
↓	↓	Endotoxemia
↓	?	Blister beetle poisoning
↓	↓	Lactation tetany in Shetland mares
↓	↓	Refeeding syndrome
↓	↓	Pamidronate therapy
See text		Other potential causes and contributors

* A relatively common disorder, condition, or mechanism
[a] Arrows indicate which values can become decreased with each disorder, condition, or mechanism.
[b] A ↓ [tMg^{2+}] might occur if the exposure to anions is great enough.

B. Inadequate ruminal or intestinal absorption of Mg^{2+}
   1. Prolonged anorexia, poor feed intake (especially in lactating cattle), or poor diet
      a. In one study that involved the death of 55 dairy cows, marked hypomagnesemia was present with clinical tetany (except in one cow).[284] For tetany to be associated with hypomagnesemia, concurrent hypocalcemia may be required.
      b. Serum [tMg^{2+}] and [fMg^{2+}] decreased in cats fed Mg^{2+}-deficient diets.[285]
      c. Serum [fMg^{2+}] remained unchanged when horses were fed a reduced-Mg^{2+} diet even though there was decreased renal Mg^{2+} excretion and decreased Mg^{2+} content in muscles.[286]
   2. Calves on a whole milk diet can develop hypomagnesemia because the dietary Mg^{2+} requirement of growing calves is about 50 % greater than the amount of Mg^{2+} available from milk.[55]
   3. Grass tetany in cattle[55,271]

a. The hypomagnesemia and free hypomagnesemia are considered to result from decreased ruminal absorption of Mg^{2+}.
b. A lush grass diet is high in PO_4 and K^+ content, low in Mg^{2+} and Na^+, and generates a more alkaline rumen (salivary HCO_3^-, NH_4^+ from nitrogen metabolism) that decreases Mg^{2+} solubility and increases Mg^{2+} binding to ingested anions.[163] Reduced Mg^{2+} solubility and decreased intake limit absorption of Mg^{2+}, and low ruminal $[Na^+]$ and high $[K^+]$ impair the transcellular absorption through Mg^{2+} channels.
c. The roles of aldosterone in the process are not established, but a high-K^+ and low-Na^+ diet might promote aldosterone production, Mg^{2+} excretion, and hypomagnesemia. Aldosterone would also exacerbate the low Na^+:K^+ ratio of rumen contents via a decrease in the Na^+:K^+ ratio in saliva.[287]
d. Grass tetany is difficult to diagnose postmortem because of the lack of gross or microscopic lesions. Also, the postmortem plasma samples cannot be used for detecting hypomagnesemia because the release of Mg^{2+} from cells (other than RBCs) may falsely elevate the postmortem concentration. However, the $[tMg^{2+}]$ in aqueous and vitreous humors also decreases in animals with Mg^{2+} depletion, and the postmortem $[tMg^{2+}]$ in those fluids is more stable than in plasma.
(1) If the aqueous or vitreous fluids are to be analyzed, they should be centrifuged soon after collection to remove contaminant cells and then stored at 4 °C. The viscosity of vitreous humor can interfere with accurate pipetting.
(2) The $[tMg^{2+}]$ in vitreous humor was stable up to 48 h postmortem in bovine eyes, and, with one assay, a $[tMg^{2+}] < 0.55$ mmol/L (1.3 mg/dL) was associated with antemortem hypomagnesemic tetany. If a concurrent $[K^+]$ in the vitreous humor is > 10 mmol/L, then there has been too much postmortem change to consider the $[tMg^{2+}]$ a reliable indicator of antemortem concentrations.[275]
(3) The $[tMg^{2+}]$ in aqueous humor was stable up to 24 h postmortem in bovine eyes, and, with one assay, a $[tMg^{2+}] < 0.25$ mmol/L (0.6 mg/dL) was associated with antemortem hypomagnesemic tetany.[275]

4. Enteric disorders (chronic enteropathies, enteritis, enterocolitis, intestinal strangulation, intestinal resection, ileus, colic)
a. Dogs with enteric disorders may develop free and total hypomagnesemia,[154] likely because of decreased intestinal Mg^{2+} absorption and increased fecal Mg^{2+} loss, or concurrent hypoproteinemia ($\downarrow [fMg^{2+}]$). The same processes are expected in other monogastric species. In diarrheal states, altered concentration gradients may promote Mg^{2+} loss via passive movement of Mg^{2+} into the intestinal lumen.[269]
b. Horses with enterocolitis[288] and colicky horses[289] frequently have hypomagnesemia or free hypomagnesemia. Decreased intake, decreased intestinal absorption, and increased intestinal loss may be contributing factors.
c. In horses, free hypomagnesemia was common prior to and after colic surgery.[192]
d. In many cases, endotoxemia or sepsis may contribute to hypomagnesemia (see below).

C. Excess urinary excretion of Mg^{2+}
1. Osmotic diuresis causes decreased tubular resorption due to solvent drag, a major contributor to hypomagnesemia in diabetes mellitus; in cats with nonketotic diabetes mellitus or ketotic diabetes mellitus, the $[fMg^{2+}]$ was decreased more frequently than the $[tMg^{2+}]$.[290]
2. Ketonuria causes decreased tubular resorption of Mg^{2+} because of complexing with ketone bodies (β-hydroxybutyrate and acetoacetate). However, dogs with diabetic

ketoacidosis tended to have a greater [fMg^{2+}] than did dogs with uncomplicated diabetes mellitus or healthy dogs; the reason for the greater [fMg^{2+}] was not determined.[291]

3. Fluid therapy has effects similar to osmotic diuresis: an increased flow rate within renal tubules impairs fMg^{2+} resorption and may cause mild hypomagnesemia if chronic.
4. Diuretics
   a. Loop diuretics inhibit the Na^+-K^+-$2Cl^-$ cotransporter in the thick ascending limb of the loop of Henle, thus impairing Mg^{2+} resorption; hypomagnesemia is typically mild, likely because volume contraction promotes more resorption in the proximal tubules.
   b. Thiazide diuretics downregulate distal tubular TRMP6 expression and inhibit the distal tubular Na^+-Cl^- cotransporter that facilitates active transport of Mg^{2+} through TRMP6 channels.
   c. Carbonic anhydrase inhibitors reduce conservation of HCO_3^- in the proximal convoluted tubule, thus reducing Na^+ and H_2O resorption leading to increased tubular flow rates and decreased Mg^{2+} resorption.
5. Other therapeutic agents affecting renal tubule function: aminoglycoside antibiotics, cisplatin, carboplatin, cyclosporine, amphotericin B, calcineurin inhibitors, and epidermal growth factor inhibitors[268,269,292]
   a. These drugs are recognized as common causes of hypomagnesemia in human patients; similar outcomes are possible in domestic mammals.
   b. Aminoglycoside antibiotics are thought to stimulate the CaSR in the thick ascending limb of the loop of Henle, thereby impairing paracellular Mg^{2+} resorption.
   c. The other listed drugs impair transcellular Mg^{2+} resorption through effects on TRMP6 ion channels in the distal convoluted tubule.

D. Mg^{2+} binding to anions
1. Citrate: Excessive citrate can transiently decrease [fMg^{2+}]; for example, [fMg^{2+}] decreased approximately 45 % in dogs undergoing plateletpheresis with citrate anticoagulation.[180] Such effects may be increased with hepatic dysfunction because of reduced capacity to metabolize citrate.
2. Fatty acids: Decreased [fMg^{2+}] may occur when Mg^{2+} binds to fatty acid anions in plasma and adipocytes during lipolytic conditions.[293] The [tMg^{2+}] may also decrease by removal of these insoluble complexes from circulation or by fMg^{2+} shifting into adipocytes down a concentration gradient caused by fMg^{2+} binding to intracellular fatty acid anions.[294] There are many potential causes of increased [fatty acids], including lipolysis induced by anorexia or endogenous or exogenous catecholamines.

E. Other or unknown mechanisms
1. Endotoxemia in horses causes hypomagnesemia as well as hypocalcemia. During experimental endotoxemia, both [tMg^{2+}] and [fMg^{2+}] were decreased despite decreased urinary Mg^{2+} excretion. The changes occurred quickly and were thought to be due to movement of Mg^{2+} from plasma to cells, potentially related to actions of insulin or vasopressin.[186] Endotoxemia may contribute to hypomagnesemia that has been associated with a variety of gastrointestinal disorders. Experimental endotoxemia did not cause hypomagnesemia in a canine study[188].
2. Blister beetle poisoning:[206,207] Hypomagnesemia from cantharidin toxicity has been reported in horses and an alpaca. The pathogenesis has not been documented, but a loss of protein-bound Mg^{2+} can contribute.
3. Lactation tetany in Shetland mares[165]
4. Refeeding syndrome: Hypomagnesemia accompanied by hypophosphatemia and hypokalemia has been reported in cats with refeeding syndrome.[295] It is thought that the

sudden increase in carbohydrate metabolism upon initiation of nutritional therapy causes hyperinsulinemia and consequent shifting of Mg^{2+}, K^+, and PO_4 into cells. Total body Mg^{2+} depletion associated with poor nutrition may contribute.

5. Pamidronate, a bisphosphonate prescribed to impair osteolysis (e.g., in certain hypercalcemic patients), is associated with hypomagnesemia in a small percentage of patients.[296] The effect may relate to shifting of Mg^{2+} to bone after total body Mg^{2+} depletion induced by underlying disease (e.g., hypercalcemia).
6. In people, hypomagnesemia has also been associated with other conditions that may affect domestic mammals. These include several mutations causing defects in active or passive Mg^{2+} resorption,[292] postobstructive diuresis, the diuretic phase of acute tubular necrosis, acute polyuric leptospirosis, treatment with pentamidine (renal tubular damage), hungry bone syndrome after parathyroidectomy or total thyroidectomy in hyperthyroid patients,[297] thyrotoxic periodic paralysis,[298] treatment with the antiviral foscarnet (a pyrophosphate analog that binds cations including fMg^{2+}), proton pump inhibitors, chronic asthma, and use of β2-agonists.
7. Factors that may contribute to hypomagnesemia in a variety of conditions include insulin administration,[299] correction of chronic metabolic or respiratory acidosis,[300] tissue reperfusion, epinephrine administration, acidemia, and hypercalcemia. Acidemia promotes hypomagnesemia by decreasing the number of Mg^{2+}-resorbing TRMP6 channels in the distal tubules. Increased $[fCa^{2+}]$ inhibits paracellular Mg^{2+} resorption in the thick ascending limb of the loop of Henle and may promote a shift of Mg^{2+} from protein and NPA in blood as Ca^{2+} replaces Mg^{2+}, thus decreasing $[tMg^{2+}]$ more than $[fMg^{2+}]$.[301]

F. Effects of hypomagnesemia on $[fCa^{2+}]$ and $[K^+]$
1. Hypomagnesemia can cause hypocalcemia by impairing PTH synthesis and secretion and therefore decreasing 1α-hydroxylase activity leading to less $1,25(OH)_2D$ formation. Decreased PTH and 25OHD causes less mobilization and intestinal absorption of Ca^{2+} (and PO_4) and decreased renal PO_4 resorption. In addition, end-organ resistance to PTH induced by hypomagnesemia has been described.[302]
2. Hypomagnesemia can cause hypokalemia because it is a cofactor for Na^+-K^+-adenosine triphosphatase (ATPase) cell membrane pumps that transport K^+ into cells; impaired K^+ entry into cells leads to increased urinary excretion of K^+.

PARATHYROID HORMONE (PTH) CONCENTRATION

I. Physiologic processes

A. *PTH* is a polypeptide hormone (84 amino acids) produced by parathyroid glands and inactivated or degraded mostly by kidneys and liver. PTH is secreted by parathyroid glands in response to a decreased $[fCa^{2+}]$ in blood, whereas vitamin D and an increased $[fCa^{2+}]$ inhibit synthesis of PTH.
1. PTH secretion is primarily mediated by fCa^{2+} binding to the parathyroid gland's chief cell CaSR. This receptor is linked to a series of intracellular signaling pathways that inhibit secretion of preformed PTH within minutes.[303,304] Mutations in the CaSR gene can cause increased or decreased receptor activity and hypocalcemia or hypercalcemia, respectively.
2. Mg^{2+} also binds to the CaSR but has less effect than Ca^{2+}. With hypermagnesemia, Mg^{2+} activates the CaSR and suppresses PTH secretion, particularly when $[fCa^{2+}]$ is low. Somewhat paradoxically, hypomagnesemia inhibits PTH secretion, likely through effects on components of the signaling pathway.[305]

3. The acid-base status of an animal also affects the secretion of PTH. Decreases in pH caused by metabolic or respiratory acidoses stimulate PTH secretion, although, in metabolic acidosis, the increase in [fCa^{2+}] due to pH-induced shifts can be sufficient to inhibit increases in PTH. Increases in pH caused by alkaloses (metabolic and respiratory) inhibit PTH secretion.[306,307] The altered [PTH] in acid-base disorders may reflect its roles in maintaining acid-base status. The PO_4 released from bone acts as a buffer in blood, and the PO_4 participates in the renal excretion of H^+. Also, persistently increased PTH activity reduces the renal excretion of HCO_3^-.[308]

B. Primary target organs: bone, intestine, and kidney
   1. PTH indirectly promotes increased intestinal Ca^{2+} absorption by promoting renal 1α-hydroxylase activity (not in horses) and formation of $1,25(OH)_2D$.
   2. PTH directly promotes increased Ca^{2+} resorption in renal tubules (Figure 11.1). PTH indirectly promotes renal distal tubular Ca^{2+} resorption.
   3. PTH promotes mobilization of Ca^{2+} and PO_4 from bone (Figure 11.1).
   4. PTH promotes renal PO_4 excretion by inhibiting resorption of PO_4 by tubules (a potent phosphaturic action) (Figure 11.6).

C. The net effect of PTH activity is to increase plasma [fCa^{2+}] by Ca^{2+} mobilization and decrease plasma [Pi] by promoting phosphaturia.

D. PTH fragments secreted from the parathyroid gland and formed from hepatic degradation of intact PTH circulate at greater concentrations than intact PTH itself. Fragments lacking the N-terminus lack classic activity of PTH, though they may not be biologically inactive. With renal failure, decreased GFR and decreased protein catabolism by renal tubular epithelial cells increase concentrations of PTH fragments in plasma.

II. Analytical concepts for PTH assays

A. RIAs developed to detect human PTH have sufficient species cross-reactivity to be valid for detecting PTH in domestic mammals, and they do not detect PTHrP.[56] Early assays included detection of PTH fragments lacking the functional N-terminal domain, but second and third generation assays for "intact" and "whole" PTH, respectively, were designed to exclude more of the PTH fragments lacking classic PTH activity (Figure 11.8). Recent and current assays have generally yielded results that fit clinical findings in domestic species. Because most PTH immunoassays detect various PTH fragments in addition to the complete PTH polypeptide, the analyte of these assays can collectively be called *immunoreactive PTH*.[309] In this book, *[PTH]* denotes immunoreactive parathyroid hormone concentration; the specific PTH fragments detected by various assays in healthy and diseased veterinary species are not characterized.

B. Assays previously and currently used in veterinary medicine include human RIAs, chemiluminescent immunoassays, enzyme-linked immunosorbent assays, and fluorescence immunoenzymatic assays, but information about analytical validation and comparison of current assays in veterinary species is limited.[154,310–312] A human immunoradiometric assay for whole and/or intact human PTH was validated and used in horses,[313] dogs,[310,314] and cats.[315] Chemiluminescent assays have varied in their performance, some being considered suitable and others unsuitable for clinical use in dogs.[310] The magnitude of reported results can vary substantially with the type of assay.

C. Samples for [PTH]
   1. Samples should be collected from dogs after an overnight fast to avoid lipemia. Also, postprandial alkalosis and variations in the [fCa^{2+}] after meals can alter the [PTH] in dogs.[39] Similar changes might occur in cats and horses.

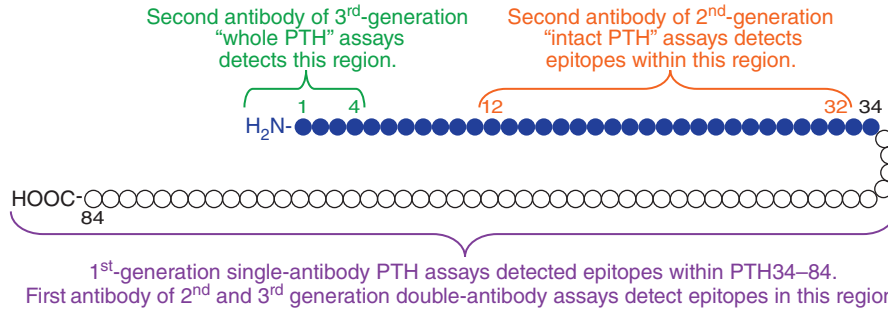


Figure 11.8. Parathyroid hormone (PTH) and PTH immunoassays.

- The solid blue circles represent amino acids 1–34 of the 84 amino acids in PTH, the PTH segment required for classic biological activity in bone and kidneys.
- First-generation assays were single-antibody competitive assays that used antibodies targeting epitopes within PTH34–84, the most immunogenic portion of PTH but lacking the N terminus and classic biologic activity. They detected many nonfunctional PTH fragments.
- Second-generation assays reduce detection of nonfunctional PTH fragments by capturing PTH's carboxyl terminal (first antibody) and then detecting only those captured fragments that contain epitopes nearer the amino-terminal (second antibody), thus excluding fragments with the carboxyl region but lacking the targeted detection region. These assays were named "intact PTH" assays (for entire PTH molecule), but they detect some PTH fragments lacking the entire PTH1–34 functional region.
- Third-generation assays also use a first antibody to capture PTH molecules by their carboxyl terminals, but the second antibody used to detect PTH requires the presence of PTH1–4. These assays are called "bioactive PTH" or "whole PTH" assays.

2. Serum is the most common sample in clinical use, and it allows concurrent measurement of [fCa^{2+}] or normalized [fCa^{2+}]. However, because PTH is susceptible to proteolysis, the preferred sample for PTH is EDTA-plasma that contains a protease inhibitor (aprotinin), and plasma should be separated from cells prior to shipping to a laboratory. Serum [PTH] remained stable at 21 °C and 37 °C for 2 d with two protease inhibitors (Pefabloc SC and 4-[2-aminoethyl]-benzenesulfonyl fluoride), but it decreased with other protease inhibitors.[316] If the sample is not analyzed on the day of collection, some investigators write that plasma or serum without aprotinin is acceptable if the sample remains frozen during storage or transit.[317]
3. Stability studies of [PTH] in pooled canine serum showed mild decreases (< 10 %) by 4 h at room temperature and by 24 h at 4 °C, with progressively greater decreases thereafter.[311] For pooled feline serum, [PTH] decreased by 21 % and 14 % by 6 h after storage at room temperature and 4 °C, respectively.[312]
4. The [PTH] in equine EDTA plasma was stable for up to 3 mo when stored at –20 °C.[318] The [PTH] of pooled canine serum decreased < 10 % by 6 mo of storage at –20 °C,[311] but the decrease in feline pooled serum stored at –20 °C was 8 % at 7 d and 23 % at 30 d.[312]

D. The plasma or serum [fCa^{2+}] or, in some cases, the serum [tCa^{2+}] of the same sample must be known to interpret the [PTH]. For example, a high-normal or increased [PTH] in the presence of hypercalcemia would represent a pathologic state, whereas, in the presence of hypocalcemia, it may represent a physiologic response.

Table 11.8. Disorders or conditions that cause increased or inappropriately high [PTH]
Increased PTH production by neoplastic cells
Neoplastic parathyroid gland (primary hyperparathyroidism)
Multiple endocrine neoplasia (type 1 or 2A)
Increased PTH production by hyperplastic parathyroid glands (idiopathic or secondary hyperparathyroidism)[a]
Chronic or acute renal failure*
Diet with a low Ca^{2+}:PO_4 ratio
Hypocalcemic disorders (with a decreased [fCa^{2+}]) other than hypoparathyroidism* (e.g., chronic enteropathies in dogs, hereditary rickets, milk fever)
Pseudohypoparathyroidism (decreased PTH receptor responsiveness)
Hyperadrenocorticism in dogs

* A relatively common disorder, condition, or mechanism

[a] These disorders are discussed in relevant portions of the hypocalcemia section of this chapter.

III. Increased or inappropriately high serum or plasma [PTH] (Table 11.8 and Figure 11.9)
   A. For most disorders that cause increased serum or plasma [PTH], increases in [PTH] are due to excessive production of PTH by neoplastic parathyroid cells or by hyperplastic parathyroid cells (secondary to decreased [fCa^{2+}]) (Table 11.8). These disorders are discussed in relevant portions of the hypocalcemia section of this chapter.
   B. Many dogs with hypercalcemia due to primary hyperparathyroidism do not have an increased [PTH]. Instead, the [PTH] is WRI but inappropriately high (upper half of the RI) in relation to an increased [fCa^{2+}], thus reflecting defective negative feedback on PTH secretion.
   C. In one study, many dogs with hyperadrenocorticism were normocalcemic with unexplained increases in plasma [PTH].[219] In another study, dogs with hyperadrenocorticism had increased [PTH] and were found to be hypercalciuric, a few with free hypocalcemia.[222] These findings support secondary adrenal hyperparathyroidism. Changes in [PTH] were not detected in a study of dogs with pituitary-dependent hyperadrenocorticism.[220]

IV. Decreased or inappropriately low serum or plasma [PTH] (Table 11.9 and Figure 11.9)
   A. Decreased PTH production occurs with decreased functional parathyroid mass (damage, removal) or feedback inhibition from increased [fCa^{2+}] that is not mediated by PTH (Table 11.9). These disorders are discussed in relevant portions of the hypocalcemia and hypercalcemia sections of this chapter.
   B. If [fCa^{2+}] is decreased and [PTH] is WRI but in the lower half of the RI, that would suggest that there has not been an appropriate PTH response.

PARATHYROID HORMONE–RELATED PROTEIN (PTHrP) CONCENTRATION

I. Physiologic processes
   A. PTHrP promotes Ca^{2+} balance in the fetus and modulates cartilage and bone development. After birth, the control of Ca^{2+} balance switches from PTHrP to PTH.[56]
   B. PTHrP is produced by many cells in fetuses and adults, but plasma [PTHrP] is very low in healthy adults. Increases in plasma [PTHrP] are detectable, but decreases are not.

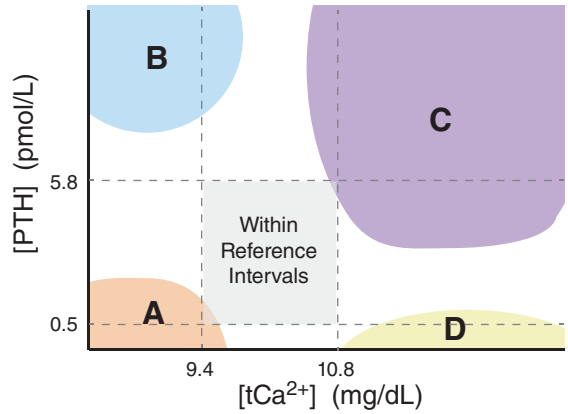


Figure 11.9. Relationships between [PTH] and [tCa^{2+}] in selected disorders of calcium homeostasis. Interpretation of a [PTH] requires a concurrent [fCa^{2+}] or [tCa^{2+}], because ↑ or ↓ [PTH] can occur with either hypercalcemia or hypocalcemia. An accurate [fCa^{2+}] is preferable to [tCa^{2+}] because the latter may be decreased simply because of hypoproteinemia or increased because of increases in NPA-bound Ca^{2+}. Other clinical and laboratory findings may be helpful. This graph schematically depicts regions in which paired results for [PTH] and [tCa^{2+}] would be expected for the selected disorders when [tCa^{2+}] is not significantly affected by changes in protein or NPA concentrations. Dashed lines represent one laboratory's lower and upper reference limits for [PTH] and [tCa^{2+}], and the intersection of the two RIs defines the region in which values from healthy individuals would be expected. The following A–D comments correspond to regions A–D.

A. Decreased PTH production due to damaged or removed parathyroid glands (hypoparathyroidism): [PTH] is ↓ or WRI but too low to indicate that the parathyroid glands are responding to decreased [fCa^{2+}]; therefore, the findings support hypocalcemia from decreased parathyroid gland function. Hypomagnesemia may yield values in region A as well.

B. Increased PTH production by hyperplastic parathyroid glands (idiopathic or secondary hyperparathyroidism such as chronic renal disease or a diet with a low Ca^{2+}:PO_4 ratio): In this case, hypocalcemia is accompanied by an ↑ [PTH], indicating that the parathyroid glands are responding to free hypocalcemia rather than causing it. [PTH] is usually quite high when secondary renal or nutritional hyperparathyroidism is recognized. In vitamin D deficiency, paired values would also tend toward the upper left, but the [PTH] would typically be lower and hypocalcemia not as marked. Paired values from other disorders causing free hypocalcemia without diseased parathyroid glands, including pseudohypoparathyroidism, would also be expected to fall within the upper left quadrant.

C. Increased PTH production by neoplastic cells (e.g., primary hyperparathyroidism): The combination of ↑ [PTH] and ↑ [tCa^{2+}] indicates that PTH is responsible for the hypercalcemia. Note that a [PTH] in the upper portion of the RI would not be expected with a physiologic response to hypercalcemia, so such paired values support hyperparathyroidism despite the [PTH] not being increased.

D. Decreased PTH production due to inhibition caused by PTH-independent free hypercalcemia (e.g., hypervitaminosis D, increased [PTHrP]): A decreased or low-normal [PTH] is an appropriate response to hypercalcemia, indicating healthy parathyroid glands and a different cause of hypercalcemia.

II. Analytical concepts
   A. Sample
      1. Because of rapid proteolysis of PTHrP, it has been recommended that blood be collected into EDTA tubes with an added protease inhibitor (aprotinin or leupeptin). Plasma should be separated within 1 h and either analyzed fresh or frozen until shipment on ice to a reference laboratory.[319]
      2. Serum can yield falsely decreased results, likely from PTHrP degradation.

Table 11.9. Disorders or conditions that cause decreased or inappropriately low [PTH][a]
Decreased PTH production due to damaged or removed parathyroid glands (hypoparathyroidism)
Decreased PTH production due to inhibition
Hypervitaminosis D
Hypercalcemic disorders (with an increased [fCa^{2+}])* except primary hyperparathyroidism
Hypomagnesemia due to Mg^{2+} depletion

* A relatively common disorder, condition, or mechanism

[a] These disorders are discussed in relevant portions of the hypocalcemia and hypercalcemia sections of this chapter.

3. Hemolysis should be avoided because it may result in falsely increased results. The package insert for one PTHrP assay kit (Beckman Coulter PTHrP IRMA) indicates that hemolyzed, icteric, and lipemic samples should be avoided because they can yield false values, but details were not found.
4. When PTHrP is assessed with PTH and fCa^{2+}, sample submission guidelines for all three tests must be considered.

B. [PTHrP] is measured by RIAs designed to measure human [PTHrP]. Clinical and laboratory findings support that some human assays can detect increased [PTHrP] when present in at least some dogs, cats, and horses;[64,66] however, one N-terminal assay did not appear valid for equine [PTHrP].[56]

III. Increased [PTHrP] in plasma

A. Some neoplasms (especially some lymphomas and carcinomas) produce enough PTHrP to cause an increase in plasma [PTHrP]. The increased PTHrP activity can result in hypercalcemia and hypophosphatemia by its actions on major target tissues (Figure 11.4). There also are rare reports of increased [PTHrP] in dogs with granulomatous diseases.[82]

B. More information about these hypercalcemic and sometimes hypophosphatemic disorders is presented elsewhere in this chapter (see Total and Free Calcium (tCa^{2+}, fCa^{2+}) Concentrations).

VITAMIN D CONCENTRATION

I. Physiologic processes

A. Formation and forms of vitamin D

1. The term *vitamin D* generally encompasses a group of similar steroid-like molecules (secosteroids) that are critical for intestinal absorption of Ca^{2+}, PO_4, and Mg^{2+}. The various forms are referred to by multiple and inconsistent terms. Some common names and structural relationships of the major forms are provided in Figure 11.10.
   a. The two major classes of vitamin D are vitamin D2 and vitamin D3 (traditionally written D_2 and D_3); there is no D1. *Vitamin D2* is ergocalciferol, but the term is also used to include ergocalciferol's metabolites. *Vitamin D3* is cholecalciferol, but the term is also used to include cholecalciferol's metabolites.
   b. Differences in the side chains of vitamins D2 and D3 result in differences in their kinetics and metabolism, but their major active metabolites $1,25(OH)_2D2$ and $1,25(OH)_2D3$ have comparable effects on calcium homeostasis through the vitamin D receptor.[320]

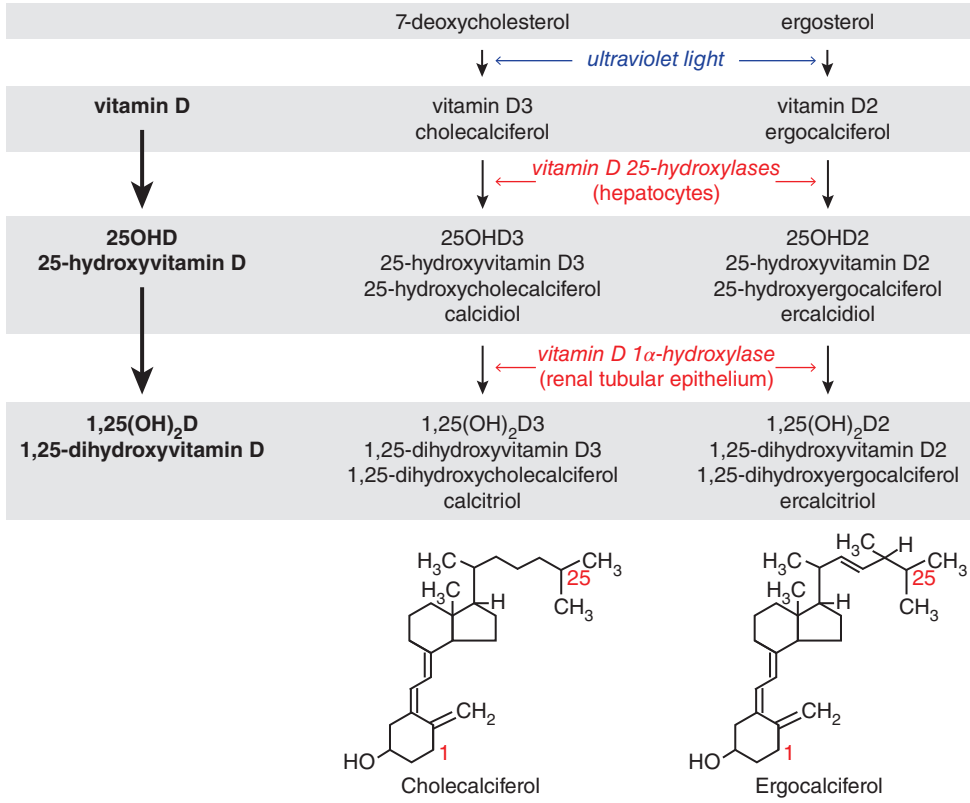


Figure 11.10. Names and structures of the major forms of vitamin D.

- Ultraviolet light and heat convert certain sterols associated with plants or in the skin of some mammals to vitamin D: ergocalciferol (vitamin D2) forms from ergosterol; cholecalciferol (vitamin D3) forms from 7-deoxycholesterol. As shown, these secosteroids (one of the four steroid rings is split open) have different side chains.
- In tissues, especially hepatocytes, ergocalciferol and cholecalciferol undergo hydroxylation (OH addition) at carbon 25 (see chemical structures) to form 25OHD – 25OHD2 or 25OHD3 – which have limited vitamin D activity. The 25OHD produced from cholecalciferol is also called calcidiol or calcifediol, and that produced from ergocalciferol is called ercalcidiol. (However, the term *calcidiol* is also used collectively for both forms of 25OHD).
- 1α-hydroxylation then adds another hydroxyl group at carbon 1 (see chemical structures) to form $1,25(OH)_2D$ – $1,25(OH)_2D2$ or $1,25(OH)_2D3$ – the most potent forms of vitamin D. This hydroxylation occurs mostly in renal tubular epithelial cells, except in horses; horses lack renal 1α-hydroxylase activity. The $1,25(OH)_2D$ derived from cholecalciferol is commonly called calcitriol, and that produced from ergocalciferol is called ercalcitriol. (However, the term *calcitriol* is also used collectively for both forms of $1,25(OH)_2D$.)

2. Sources and metabolism of vitamin D (Figure 11.11)
   a. Dietary intake of inactive vitamin D2 or D3 is important for most domestic mammals, and necessary for dogs and cats. It is usually provided as a daily dietary supplement, but it is also ingested in natural diets, either as ergocalciferol (primarily in fungi or fungus-contaminated plants) or as cholecalciferol (primarily in fatty fish or in liver, fat, or certain other tissues of prey, but also in some plants).[91] Because vitamin D is fat soluble, bile acids promote its absorption.

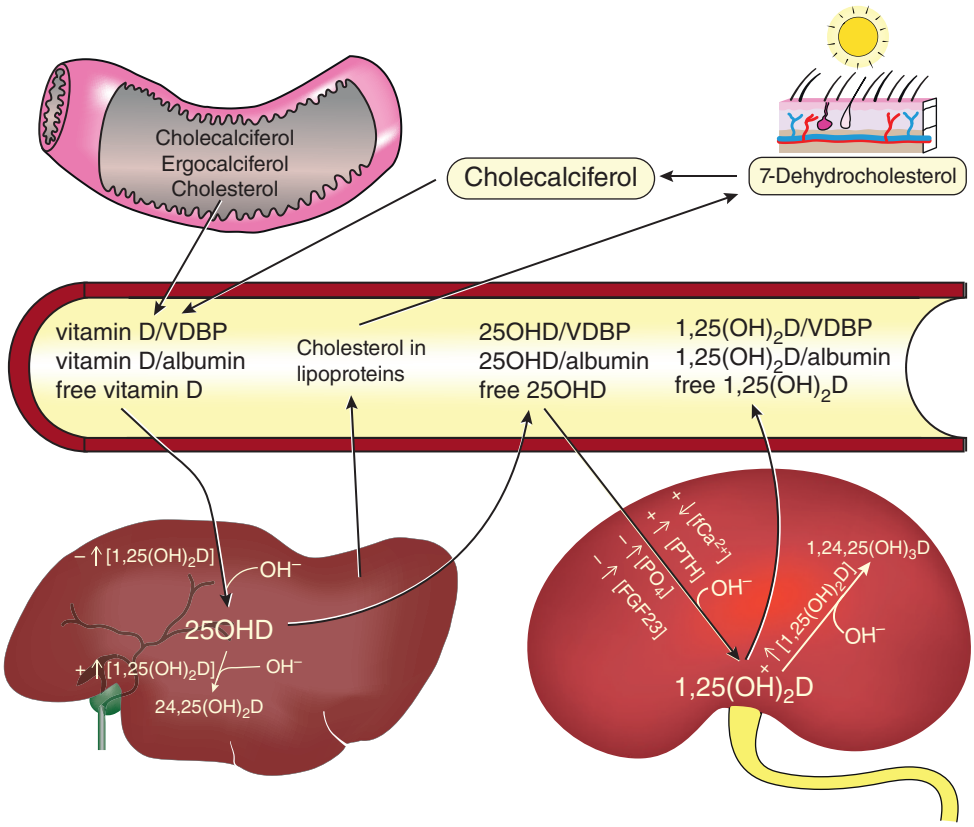


Figure 11.11. Sources and metabolism of Vitamin D.

- Various forms of vitamin D in natural or supplemented diets are ingested and absorbed.
- In cutaneous epithelial cells of some species (see the text), cholecalciferol is synthesized from 7-deoxycholesterol derived from dietary or endogenous cholesterol.
- Cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) enter blood where they are mostly bound to VDBP. Most of the vitamin D is extracted from blood by hepatocytes and hydroxylated to produce 25OHD3 (from cholecalciferol) or 25OHD2 (from ergocalciferol).
- Most circulating 25OHD is tightly bound to VDBP; < 0.1 % is free and diffuses into cells throughout the body where it can be further metabolized or affect cell functions directly. Both free and bound 25OHD pass freely through the glomerular filtration barrier and are reclaimed by proximal tubular epithelial cells, which convert 25OHD to $1,25(OH)_2D$ by vitamin D 1α-hydroxylase (CYP27B1) (except in horses – see the text); this ultimate active form of vitamin D has local and systemic effects. Renal 1α-hydroxylation is highly regulated; ↓ $[fCa^{2+}]$ and ↑ [PTH] promote it, whereas ↑ $[PO_4]$ and ↑ [FGF23] inhibit it.
- Control of vitamin D activity also occurs through the catabolic actions of enzymes including vitamin D 24-hydroxylase, which is highly induced by increased $1,25(OH)_2D$ (negative feedback) to act on both $1,25(OH)_2D$ and 25OHD, thus forming $24,25(OH)_2D$ (mostly in the liver) and $1,24,25(OH)_3D$ (mostly in the kidneys). These forms have reduced vitamin D activity and undergo further catabolism. In the kidney, inactivation through 24 hydroxylation is also promoted by ↑ [FGF23] and ↑ $[PO_4]$ (not shown); it is inhibited by ↑ [PTH] and ↓ $[fCa^{2+}]$ (not shown).
- Like vitamin D and 25OHD, plasma $1,25(OH)_2D$ is mostly bound to VDBP, with less bound to albumin, and < 1 % as free molecules.

$1,24,25(OH)_3D$, 1,24,25-trihydroxycholecalciferol; 24,25-DHCC, 24,25-dihydroxycholecalciferol; CYP, cytochrome P450 oxidase

b. Sunlight generates cholecalciferol from cholesterol-derived 7-deoxycholesterol in cutaneous squamous epithelial cells of some domestic animals, but to a variable degree. This pathway occurs in ruminants, pigs, and camelids, but it appears limited in cattle[145] and horses;[18] it does not occur significantly in dogs and cats.[146,147].

c. After intestinal absorption or cutaneous synthesis, unregulated 25-hydroxylation and highly regulated 1α-hydroxylation yield hormonally active vitamin D metabolites: 25OHD and $1,25(OH)_2D$, the latter having a thousandth the plasma concentration of the former, but much greater activity (considered the active form). Several enzymes in cells throughout the body can metabolize vitamin D and produce various active forms with local effects, but the hepatorenal pathway illustrated in Figure 11.11 appears to be largely responsible for plasma concentrations of 25OHD and $1,25(OH)_2D$ in most healthy mammals.

(1) Hepatic 25-hydroxylation is mediated by several enzymes, but mostly by vitamin D 25-hydroxylase (CYP2R1), which appears to function without hormonal regulation. Because this production is mostly unregulated, plasma [25OHD] is a reasonable reflection of vitamin D status (intake and production).

(2) Ultimate activation of vitamin D to its most active form, $1,25(OH)_2D$, occurs in mitochondria of proximal tubular epithelial cells via 1α-hydroxylation of 25OHD reclaimed from the renal filtrate by megalin/cubilin-mediated endocytosis.[148]

(3) Horses lack renal 1α-hydroxylase activity and have low plasma concentrations of $1,25(OH)_2D$.[17,18] They become hypercalcemic in response to vitamin D3 injections, but without measurable increases in $[1,25(OH)_2D]$. Overall plasma [25OHD] is low compared to other domestic mammals; 25OHD2 appears to predominate and 25OHD3 concentrations are undetectable to low. They also have low concentrations of plasma $24,25(OH)_2D3$. However, they have a greater intestinal capacity to absorb Ca^{2+} without vitamin D, and they have greater renal excretion of Ca^{2+} than other domestic mammals.

d. In people, 85–90 % of vitamin D and its metabolites circulate in plasma bound to VDBP, a well-conserved protein somewhat smaller than albumin (relative molecular mass = 50,000–60,000). Most of the rest is bound to albumin, and very little is free (< 1 %).[321,322] $1,25(OH)_2D$ dissociates from VDBP much more readily than does 25OHD, so it moves to cells more easily and has a half-life in people of just a few hours compared to the 2–3 wk half-life of 25(OH)D. Vitamin D itself has a half-life of up to a day. In species that have been studied, the vitamin D2 forms have lower affinity for VDBP than their respective D3 forms, and that contributes to shorter half-lives. VDBP also transports actin and fatty acids.

e. Catabolism of 25(OH)D and $1,25(OH)_2D$ helps limit vitamin D activity in cells throughout the body. Enzymatic alterations generate various vitamin D forms with less and varied activity, differing affinities for VDBP, and different plasma half-lives. The first major step is 24-hydroxylation via vitamin D 24-hydroxylase (CYP24A1) (Figure 11.11). Its importance is evidenced by people with *CYP24A1* mutations who develop hypercalcemia, decreased [PTH], and high-normal to increased $[1,25(OH)_2D]$.[323]

3. Regulation of vitamin D metabolism occurs through control of the catabolism and production of $1,25(OH)_2D$ (Figure 11.11). Increased [PTH] and decreased $[fCa^{2+}]$ promote renal 1α-hydroxylation and inhibit 24-hydroxylation, thus increasing $[1,25(OH)_2D]$, whereas increased [FGF23] and increased $[PO_4]$ inhibit 1α-hydroxylation, promote 24-hydroxylation, and therefore decrease $[1,25(OH)_2D]$. 1α-hydroxylation occurring in other cell types, such as macrophages, is not regulated in this way; instead, it is responsive to various cytokines such as interferon, TNF, and IL-1.[324]

B. Effects of vitamin D: Vitamin D (mostly $1,25(OH)_2D$, but not in horses) mediates most of its effects by binding to the vitamin D receptor, a nuclear hormone receptor and transcription factor present in most cells of the body. Binding to this receptor leads to up- or downregulation of genes, including those related to calcium homeostasis in bone, intestine, and kidney. Vitamin D also has rapid nongenomic effects on certain cells. Actions of $1,25(OH)_2D$ related to calcium homeostasis in dogs, cats, and cattle include stimulatory and inhibitory roles (Figure 11.1).
   1. Vitamin D promotes intestinal uptake of Ca^{2+} by increasing calmodulin in the brush border of mucosal epithelial cells and inducing these cells to form the components involved in transcellular transport: Ca^{2+} channels, Ca^{2+}-binding protein (calbindin), and a Ca^{2+} adenosine phosphatase (ATPase) pump. Vitamin D also stimulates absorption of PO_4 and Mg^{2+} from the intestinal lumen.
   2. Vitamin D has complex direct and indirect effects on bone, a major indirect effect being the delivery of Ca^{2+} and PO_4 to bone by promoting absorption from the intestine. It also promotes osteoblast activity, chondrocyte activity, and, like PTH, stimulates osteoclastogenesis via osteoblasts, but effects on osteoclastic activity are unclear.[21]
   3. Vitamin D promotes distal renal tubular resorption of fCa^{2+} by increasing expression of Ca^{2+} channels, stimulating the formation of calbindin, and increasing the activity of the Ca^{2+} pump, all of which are involved in transcellular Ca^{2+} transport.[268]
   4. Vitamin D inhibits PTH synthesis in parathyroid glands by inhibiting transcription of the PTH mRNA.
   5. Net major effect of vitamin D on calcium homeostasis: promotion of hypercalcemia

Note: Roles of vitamin D metabolites in regulation of $[fCa^{2+}]$ in horses are different and appear to be reduced compared to their roles in other species.[17,18]

II. Analytical concepts
   A. Sample
      1. Serum (harvested within an hour) is the common sample, but plasma (with either heparin or EDTA) is generally acceptable. Concentrations of $1,25(OH)_2D$ and 25OHD are stable for 3 d at 24 °C.[325] Samples should be frozen if analysis is delayed.
      2. For most current assays, acetonitrile is used to deproteinize the sample and free vitamin D metabolites from VDBP and albumin; thus, the assays measure total concentrations and not just the free concentrations, which are < 1 % of the total. Assay results may not reflect concentrations of the most biologically available forms.
   B. Assays
      1. Incomplete extraction of vitamin D from VDBP may falsely decrease results. Recovery of vitamin D metabolites from canine serum was only about 50–60 % in one report.[326]
      2. The $[1,25(OH)_2D]$ resulting from $1,25(OH)_2D3$ and $1,25(OH)_2D2$ together can be measured by a radioreceptor assay using a vitamin D receptor from calf thymus,[327] as well as RIAs and chemiluminescent assays.[328,329] Poor antibody specificity may allow detection of other vitamin D metabolites.
      3. The [25OHD] resulting from 25OHD3 and 25OHD2 together have been measured by competitive RIAs and (electro)chemiluminescence immunoassays. Assay antibodies might not react with 25OHD3 and 25OHD2 to the same degree and might cross-react with other vitamin D metabolites that circulate at similar concentrations (e.g., potentially $24,25(OH)_2D$ in dogs)[330] and at much lower (negligible) concentrations (e.g., $1,25(OH)_2D$). An ELISA has been used to measure free [25OHD] in dogs;[326] the clinical utility of [free 25OHD] is unknown.[331]
      4. In veterinary and human medicine, liquid chromatography tandem mass spectrometry and high-performance liquid chromatography have been used to measure concentrations

of specific vitamin D metabolites, including 24,25-dihydroxyvitamin D, specific cholecalciferol and ergocalciferol derivatives (e.g., 25OHD2 versus 25OHD3), and stereoisomers (C3-epimers) that might differ in biological activity and significance.[147,326,331]

5. In people, concentrations of so-called bioavailable 25OHD have been calculated and assessed for their diagnostic value, some reports supporting better correlation with some clinical disease states.[321]
   a. Although all bound 25OHD is in equilibrium with free 25OHD, *[bioavailable 25OHD]* has been defined as the sum of the concentrations of free 25OHD and 25OHD bound to albumin, thus excluding the 25OHD bound more tightly to VDBP.
   b. [Bioavailable 25OHD] has been calculated from measured [albumin], [VDBP], and [25OHD], factoring in affinity constants for the association of 25OHD with albumin and with VDBP,[331,332] but direct assays are being developed. Indirect, calculated, results are affected by method variations and inaccuracies for measuring [albumin] and [VDBP], and would require reliable species-specific affinity constants for use in veterinary medicine.
   c. The comparative utility of human assays for [total 25OHD], [free 25OHD], and [bioavailable 25OHD] is under investigation.[331]

III. Conditions and disorders related to abnormal vitamin D activity
   A. Hypervitaminosis D is created by excessive vitamin D activity. The major pathologic manifestations are hypercalcemia, hyperphosphatemia, and damage to several organs (bones, soft tissues, heart, kidneys) due to mineralization (calcification). With suspected vitamin D toxicity, the appropriate analyte to assess may be $1,25(OH)_2D$ or 25OHD, depending on the toxicant.
   B. Hypovitaminosis D is created by inadequate vitamin D activity with the major pathologic manifestations being hypocalcemia, hypophosphatemia, and impaired mineralization of bone that may lead to rickets in the young or osteomalacia in adults. Pathologic lesions of hypovitaminosis D in domestic species have been reviewed.[333] Measured concentrations of total [25OHD] or total $[1,25(OH)_2D]$ may not correlate with the deficient activity of vitamin D when carrier protein concentrations are decreased by hepatic, renal, or intestinal disease.

IV. Clinical testing for vitamin D is done for specific conditions rather than for screening. Expected results for these conditions and other potential causes of increased or decreased concentrations follow.
   A. 25OHD findings for major disorders assessed by 25OHD testing (Tables 11.10 and 11.11)
      1. General deficiencies or excesses of vitamin D are assessed by measuring [25OHD], not concentrations of the more biologically active $1,25(OH)_2D$. This is because concentrations of the latter are highly controlled and do not reflect overall vitamin D status; they may be WRI or increased secondary to PTH effects despite decreased [25OHD], or they may be WRI with hypervitaminosis D.
      2. Vitamin D toxicity: Increased [25OHD] supports hypervitaminosis D caused by excess dietary vitamin D supplementation (most forms, including salmon oil) or by cholecalciferol-containing rat poison.[93,97,98,334] The increase in [25OHD] following cholecalciferol toxicity can persist for months as cholecalciferol is released slowly from adipose tissue, where it accumulates because of its lipid solubility.[335] Measurement of [25OHD] cannot confirm toxicity from ingesting the synthetic vitamin D analog calcipotriol (calcipotriene) that is present in antipsoriasis creams (Dovonex, Sorilux). Similarly, it would not detect tacalcitol (1,24-dihydroxycholecalciferol), which is also in some antipsoriasis creams.

Table 11.10. Disorders or conditions that cause increased [25OHD]
Exogenous sources of vitamin D (hypervitaminosis D)
Excess dietary intake (misformulated ration, salmon oil supplements)
Rodenticides containing cholecalciferol

Note: The [25OHD] in young cats is lower than in adults; similar age differences may occur in other species.

Table 11.11. Disorders or conditions that cause decreased [25OHD]
Decreased ingestion or synthesis of vitamin D (hypovitaminosis D)
Decreased dietary intake of vitamin D
Insufficient exposure to sunlight (varies with species)
Intestinal malabsorption (enteropathies)
Increased renal excretion of 25OHD
Chronic renal failure
Protein-losing nephropathy
Inherited megalin/cubilin-receptor dysfunction
Other or unknown mechanisms
Sepsis/endotoxemia
Certain nonparathyroid neoplasms
Hepatic failure or cholestasis (potentially)

Note: The [25OHD] in young cats is lower than in adults; similar age differences may occur in other species.

3. Vitamin D deficiency: Decreased [25OHD] supports hypovitaminosis D. This may be caused by insufficient cholecalciferol or ergocalciferol in the diet[155] or insufficient sunlight in herbivores, particularly llamas and alpacas, which evolved in high-altitude environments with abundant solar radiation.[93] Intestinal malabsorption may limit vitamin D absorption enough to cause deficiency despite adequate amounts in food.

B. $1,25(OH)_2D$ findings for major disorders assessed by $1,25(OH)_2D$ testing
   1. Increased [$1,25(OH)_2D$] (Table 11.12)
      a. Exogenous calcitriol toxicity: This may occur from ingesting any of several synthetic $1,25(OH)_2D3$ medications or glycosides of $1,25(OH)_2D3$ in certain plants (e.g., *Cestrum diurnum*, *Solanum malacoxylon*).
      b. Endogenous nonrenal sources of $1,25(OH)_2D3$
         (1) Granulomatous inflammation: Hypercalcemia is caused by macrophage-derived $1,25(OH)_2D$ in granulomatous inflammatory disorders; macrophages have 1α-hydroxylase activity to generate $1,25(OH)_2D$ from circulating vitamin D or 25OHD, but they lack enough 24-hydroxylase activity to catabolize it.
         (2) Neoplasm-induced hypercalcemia: Rarely, some neoplasms cause hypercalcemia by producing $1,25(OH)_2D$.
      c. Hereditary $1,25(OH)_2D$–resistant rickets (vitamin D–dependent rickets type II): This rare condition is caused by a defect in the vitamin D receptor gene (see Hypocalcemia, Section B.3).[158,159]
      d. Other causes of increased [$1,25(OH)_2D$] listed in Table 11.12 relate to increased renal 1α-hydroxylase activity occurring with conditions that are typically diagnosed without $1,25(OH)_2D$ testing (see Section IV.D below).

Table 11.12. Disorders or conditions that cause increased [1,25(OH)$_2$D]
Exogenous sources of 1,25(OH)$_2$D (hypervitaminosis D)
Calcitriol medication (excess dose, accidental ingestion)
Plants containing 1,25(OH)$_2$D (*Cestrum diurnum*, *Solanum malacoxylon*)
Endogenous nonrenal sources of 1,25(OH)$_2$D
Granulomatous inflammation
Certain neoplasms
Increased renal 1α-hydroxylase activity
Primary hyperparathyroidism (parathyroid neoplasms)
Humeral hypercalcemia of malignancy caused by PTHrP (lymphoma, carcinomas)
Bovine milk fever (though the increase may be attenuated)
Vitamin D–receptor defect rickets (vitamin D–dependent rickets, type II) (rare)

Note: The [1,25(OH)$_2$D] in young cats is lower than in adults; similar age differences may occur in other species.

Table 11.13. Disorders or conditions that cause decreased [1,25(OH)$_2$D]
Decreased renal 1α-hydroxylase activity
Chronic renal failure (multiple contributing factors)*
Vitamin D–dependent rickets, type Ia (rare)
Nonrenal disease causing decreased 1α-hydroxylase activity
Primary hypoparathyroidism (damaged parathyroid gland)
Pseudohypoparathyroidism (decreased PTH receptor responsiveness)
Hypomagnesemia
Other: Sepsis/endotoxemia

* A relatively common disorder or condition

Note: The [1,25(OH)$_2$D] in young cats is lower than in adults; similar age differences may occur in other species.

3. Decreased [1,25(OH)$_2$D] (Table 11.13)
   a. Decreased renal 1α-hydroxylase activity
      (1) Chronic renal failure: Impaired production of 1,25(OH)$_2$D occurs in chronic renal failure because of reduced 25OHD availability and decreased 1α-hydroxylase activity caused by reduced renal functional mass, hyperphosphatemia, and increased FGF23 (despite stimulation of 1α-hydroxylase by increased PTH).[336] In one study, 41 dogs with chronic renal diseases had 1,25(OH)$_2$D concentrations that were progressively lower with increasing severity of azotemia.[141]
      (2) Vitamin D–dependent rickets type Ia: This rare condition is caused by a mutation in the 1α-hydroxylase gene (*CYP27B1*) such that formation of 1,25(OH)$_2$D from 25OHD is impaired.
      (3) Vitamin D–dependent rickets type Ib is a rare condition caused by a mutation in the major vitamin D 25-hydroxylase gene (*CYP2R1*) which generates 25OHD (see Hypocalcemia section B.4). It has been reported in a young cat with decreased [1,25(OH)$_2$D],[160] but [1,25(OH)$_2$D] is typically WRI or increased despite decreased [25OHD] in people with this disorder.[331]

b. Other causes of decreased [1,25$(OH)_2$D] listed in Table 11.13 relate to disorders that are typically diagnosed without 1,25$(OH)_2$D testing (see Section IV.D below).

3. [1,25$(OH)_2$D] may also be measured to monitor synthetic 1,25$(OH)_2$D3 (calcitriol) therapy.
4. Note: Assessment of [1,25$(OH)_2$D] is not indicated for suspected vitamin D (cholecalciferol or ergocalciferol) toxicity, in which case [1,25$(OH)_2$D] may be WRI or even decreased because of suppressed PTH and 1α-hydroxylase activity. However, the concentration of free 1,25$(OH)_2$D could be increased because large amounts of vitamin D or 25OHD displace it from VDBP and albumin. Testing for 25OHD would be appropriate.

C. Disorders for which [25OHD] may be decreased (Table 11.11) but that are typically diagnosed without 25OHD testing

1. Intestinal malabsorption (enteropathies): Chronic intestinal disease may substantially reduce fat and vitamin D absorption.[153,337] Dogs with inflammatory bowel disease and hypoalbuminemia suggesting protein-losing enteropathy were shown to have decreased [25OHD] associated with decreased [fCa^{2+}].[338] Other potential contributors to decreased [25OHD] include decreased intake of food, increased loss of vitamin D and its carriers (VDBP and albumin), and inflammation. In dogs with chronic enteropathy, serum [VDBP] did not differ between those with and without decreased [25OHD], suggesting that loss of VDBP is not the cause of low [25OHD]; however, there is currently no published validation of the human VDBP ELISA used in that study to confirm its applicability to canine samples.[153]
2. Chronic renal failure: In multiple studies of dogs with chronic renal failure, [25OHD] has been significantly lower than in health, whereas 1,25$(OH)_2$D results have varied.[329,337,339] Contributors to decreased [25OHD] may include decreased dietary intake of vitamin D and reduced renal functional mass resulting in decreased renal tubular resorption of 25OHD and VDBP.
3. Protein-losing nephropathy: Proteinuria may cause loss of VDBP with or without bound 25OHD, loss of 25OHD bound to albumin, and loss of 1,25$(OH)_2$D, as well as reduced expression of proximal tubular protein resorption receptors.[340,341] Compared to healthy dogs, nonazotemic dogs with protein-losing nephropathy had lower serum concentrations of 25OHD and 1,25$(OH)_2$D than healthy dogs, and their serum albumin and VDBP concentrations were correlated (source and validation information for the VDBP assay were not reported).[342] In another report, two hypocalcemic and hypoalbuminemic dogs with protein-losing nephropathies had decreased [fCa^{2+}] and decreased [25OHD]; one dog had a mildly decreased [1,25$(OH)_2$D] whereas the other's [1,25$(OH)_2$D] was WRI.[151]
4. Dogs with mutations causing megalin/cubilin-complex dysfunction and therefore diminished renal tubular resorption of VDBP, with or without bound 25OHD or 1,25$(OH)_2$D, had decreased plasma concentrations of 25OHD and 1,25$(OH)_2$D.[343]
5. Septic or perhaps nonseptic inflammation: Compared to 17 healthy dogs, 16 dogs with various causes of sepsis had significantly lower serum [25OHD].[344] Experimental endotoxemia in dogs lowered [25OHD] and [fCa^{2+}] within 2 h.[188] In people undergoing elective orthopedic surgery, [25OHD] and [VDBP] decreased as the concentration of C reactive protein, a positive acute phase protein, increased.[345] However, there was no sham control group to exclude other causes such as potential dilutional effects of fluid administration which may also decrease [25OHD].[346]

6. Some nonparathyroid neoplasms (with or without hypercalcemia): Vitamin D findings are variable in dogs with neoplasms, but groups of dogs with several types of neoplasms have been shown to have lower 25OHD concentrations than other groups.[337] Causes are likely multifactorial.[328]
7. Hepatic disease: Given the roles for the liver in vitamin D metabolism, including 25-hydroxylation of vitamin D and delivery of bile acids to the intestine to promote absorption of fat-soluble vitamins, severe hepatic dysfunction or cholestatic disease might cause or contribute to decreased [25OHD].
8. Physiologic variables: In cats, [25OHD] was lower at 3 mo of age (first age tested) than at later times up to 15 mo (last age tested).[347]

D. Other disorders that may increase or decrease [$1,25(OH)_2D$] but are not typically associated with $1,25(OH)_2D$ testing
1. Increases in [$1,25(OH)_2D$]
   a. Increased renal 1α-hydroxylase activity (Table 11.12)
      (1) PTH- or PTHrP-mediated hypercalcemia (e.g., lymphoma) including primary hyperparathyroidism and humoral hypercalcemia of malignancy or of benign disease:[57,328] PTH and PTHrP promote 1α-hydroxylase activity, which results in increased $1,25(OH)_2D$ production. In hypercalcemic dogs with various forms of HHM, there was not a consistent pattern of $1,25(OH)_2D$ concentrations; the measured [$1,25(OH)_2D$] values were decreased, WRI, or increased.[57]
      (2) Bovine milk fever: Increased PTH activity promotes increased $1,25(OH)_2D$ production via 1α-hydroxylase activity in cows with decreased [fCa^{2+}], although PTH responsiveness and effect is diminished.[161]
   b. Chronic renal failure in hypercalcemic dogs: Increased [$1,25(OH)_2D$] was reported in 2 of 10 hypercalcemic ([tCa^{2+}]) dogs diagnosed with chronic renal failure (5 in acute crisis), whereas 4 of 10 had decreased [$1,25(OH)_2D$] and none had increased [25OHD]. Reasons for the increased [$1,25(OH)_2D$] could not be determined, in part because [fCa^{2+}] was not measured.[328]
2. Decreases in [$1,25(OH)_2D$] (Table 11.13)
   a. Nonrenal disease causing decreased 1α-hydroxylase activity: Decreased 1α-hydroxylase activity decreases $1,25(OH)_2D$ production with hypoparathyroidism, pseudohypoparathyroidism, and hypomagnesemia. Hypomagnesemia also impairs PTH secretion.
   b. Sepsis/endotoxemia: Reports of [$1,25(OH)_2D$] in domestic mammals with sepsis or endotoxemia are lacking, but some people who developed hypocalcemia in association with Gram-negative sepsis had decreased [$1,25(OH)_2D$],[348] and evidence suggests that hyporesponsiveness to PTH may decrease [$1,25(OH)_2D$] production in endotoxemia.[191]
   c. Dogs with lymphoma (with or without hypercalcemia) may have $1,25(OH)_2D$ concentrations that are low, high, or WRI.[328,337]
3. Physiologic variables: In cats, [$1,25(OH)_2D$] may be greatest in kittens (3 and 6 mo of age), decreasing to adult values after that.[347]

CALCITONIN CONCENTRATION

I. Physiologic processes
   A. Calcitonin is a polypeptide hormone synthesized by thyroid C cells that are part of the APUD family of cells. (APUD is the acronym for endocrine cells that have a role in amine precursor uptake and decarboxylation.) Calcitonin forms from endopeptidase cleavage of procalcitonin. Procalcitonin can be produced by other cells in response to inflammatory cytokines and is a biomarker for sepsis.
   B. The regulation of secretion is not thoroughly understood.
      1. Calcitonin secretion is stimulated by increased concentrations of fCa^{2+} (major), fMg^{2+}, α- and β-adrenergic hormones, gastrin, and cholecystokinin.
      2. Calcitonin secretion is inhibited by decreased [fCa^{2+}] and increased somatostatin concentration.

II. Actions
   A. Calcitonin inhibits osteoclastic activity in bone.
   B. Calcitonin inhibits renal tubular resorption of Ca^{2+} and PO_4.
   C. The net effect of calcitonin activity is to decrease serum [tCa^{2+}], [fCa^{2+}], and [Pi].

III. Analytical concepts
   A. Stability studies for human serum [calcitonin] showed decreases after 2 h of storage at room temperature, and decreases by 6 h with serum storage at 4–8 °C.[349]
   B. RIAs for canine calcitonin have been developed.[350,351] Assays for human calcitonin include RIAs and chemiluminescent or fluorescent immunoassays. Assays for procalcitonin are not assays for calcitonin.
   C. Marked differences in amino acid sequences between species may limit the cross-species immunoreactivity in RIAs. Antibodies may cross-react with procalcitonin, thus changing the diagnostic specificity of the assay.[349]

IV. Increased immunoreactive calcitonin concentrations
   A. Medullary thyroid carcinoma[352]
   B. Nonthyroid cancer, especially from neural crest tissue
   C. In people, increased [calcitonin] has been documented with chronic renal failure (likely related to C-cell hyperplasia), with thyroiditis, and with use of proton pump inhibitors (e.g., omeprazole).[349]

MAJOR PATTERNS FOR CALCIUM (Ca^{2+}) AND INORGANIC PHOSPHORUS (Pi) DISORDERS

Because of the physiologic relationships among Ca^{2+}, PO_4, PTH, PTHrP, and vitamin D, some disorders are expected to cause certain patterns of abnormal analyte concentrations suggestive of those disorders. Patterns for selected disorders or conditions are listed in Table 11.14. Because of compensation, some analyte concentrations may remain WRI but be inappropriately located near either the upper or lower limits of the RI. Findings in clinical cases may represent a combination of factors. For example, an animal with primary hyperparathyroidism may have hypercalcemia because of excess PTH activity, and hyperphosphatemia because of hypercalcemic nephrosis that produced renal failure.

Table 11.14. Expected hormone and mineral patterns for selected disorders or conditions (without complications)

	[tCa^{2+}]	[Pi]	[PTH]	[PTHrP]	[25OHD]	[$1,25(OH)_2D$]
Primary hyperparathyroidism	↑[a]	↓–WRI	WRI–↑[b]	WRI	WRI	WRI–↑
HHM[c]	↑	↓–WRI	↓–WRI	↑	WRI	WRI–↑
Excess vitamin D[d]	↑	WRI–↑	↓–WRI	WRI	↑	↓–↑
Canine hypoadrenocorticism	WRI–↑	WRI–↑	↓–WRI	WRI	WRI	WRI
Renal failure, acute, hypercalcemic	↑	↑	↓–WRI	WRI	WRI	WRI
Renal failure, equine, hypercalcemic[e]	↑	WRI–↓	↓	WRI	?[f]	WRI
Renal failure, chronic[g]	↓–WRI	↑	↑	WRI	↓–WRI	↓–↑
Hypoalbuminemia	↓	WRI	WRI	WRI	WRI	WRI
Primary hypoparathyroidism	↓	WRI–↑	↓–WRI[h]	WRI	WRI	↓–WRI
Milk fever	↓	↓	WRI–↑	WRI	WRI	WRI–↑[i]
Endotoxemia	↓	↓	↑–WRI	WRI	↓–WRI	↓–WRI[j]
Prolonged anorexia	WRI	↓	WRI	WRI	WRI	WRI

[a] ↑, above the URL; ↓, below the lower reference limit
[b] The value may not be increased but is inappropriately high for the hypercalcemic status.
[c] Due to increased PHTrP production by neoplastic cells (different patterns with other hypercalcemic agents)
[d] Excess cholecalciferol or ergocalciferol, which are converted to 25OHD that may promote or suppress $1,25(OH)_2D$ production; neither [25OHD] nor [$1,25(OH)_2D$] would be increased with hypervitaminosis D caused by calcipotriol (calcipotriene, a calcitriol analog) or tacalcitol (1,24-dihydroxycholecalciferol)
[e] More common in horses than other species; can be either acute or chronic renal failure
[f] Results are not well documented; one would expect a decrease given the presence of hypercalcemia.
[g] More common than hypercalcemic renal failure in most domestic species
[h] The value may not be decreased but may be inappropriately low for the hypocalcemic status.
[i] Although the [$1,25(OH)_2D$] may be increased, the increase is limited by a state of PTH resistance.
[j] Results are not published for domestic mammals; decreases are expected based on human findings.
Note: Secondary pathologic states may alter patterns. For example, prolonged or severe hypercalcemia may cause renal failure that might change the serum [Pi] from hypophosphatemia to hyperphosphatemia.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 12

SELECTED ENZYME ACTIVITIES AND ENZYMATIC PROTEINS

Michael A. Scott, Michael M. Fry, and Steven L. Stockham

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Table 12.1. Abbreviations and symbols in this chapter	
$(GGT:Crt)_u$	Urine γ-glutamyltransferase activity to creatinine concentration
[x]	x concentration (x = analyte)
ALP	Alkaline phosphatase
ALT	Alanine transaminase[a]
AST	Aspartate transaminase[a]
ATP	Adenosine triphosphate
B-ALP	Bone alkaline phosphatase
C-ALP	Corticosteroid-induced alkaline phosphatase
CK	Creatine kinase
cPLI	Canine pancreatic lipase immunoreactivity
Crt	Creatinine
DGGR	1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester
EC	Enzyme Commission
ELISA	Enzyme-linked immunosorbent assay
GFR	Glomerular filtration rate
GGT	γ-Glutamyltransferase[b]
GMD	Glutamate dehydrogenase
GOT	Glutamate oxaloacetate transaminase[a]
GPT	Glutamate pyruvate transaminase[a]
I-ALP	Intestinal alkaline phosphatase
ID/SDH	Iditol dehydrogenase / sorbitol dehydrogenase
L-ALP	Liver alkaline phosphatase
LD	Lactate dehydrogenase
M_r	Relative molecular mass
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
P-5-P	Pyridoxal-5′-phosphate
RBC	Red blood cell, erythrocyte
RI	Reference interval
SI	Système international d'unités (International System of Units)
TG	Triglyceride
TK	Thymidine kinase
U	International unit
URL	Upper reference limit
WRI	Within reference interval

[a] The NC-IUBMB[c] recommended transaminase but considered aminotransferase acceptable.
[b] The NC-IUBMB[c] recommended γ-glutamyltransferase but considered γ-glutamyltranspeptidase (GGTP) acceptable.
[c] NC-IUBMB, Nomenclature Committee of the International Union of Biochemistry and Molecular Biology

BASIC PRINCIPLES IN CLINICAL ENZYMOLOGY

Enzymes are proteins that catalyze chemical reactions. Proteins that have different polypeptide structure but catalyze the same chemical reaction are *isoenzymes* (isozymes). If the different enzyme structure is created by a posttranslational modification of an original gene product, then the proteins are *isoforms*. Many enzymes require nonprotein cofactors to assist catalysis, converting them from *apoenzymes* (without cofactor) to *holoenzymes* (with cofactor). These are often from a vitamin source (e.g., P-5-P from vitamin B_6, NAD from niacin) but also may be ions (e.g., Ca^{2+}, Mg^{2+}).

I. Major sources of enzymes in plasma or serum (Figure 12.1 and Table 12.2)
   A. Enzymes described in this chapter are measured in plasma or serum but originate from cells and do not have recognized functions in blood. Before their release from cells, the enzymes may be in a cell's cytoplasm, mitochondria, or membrane. Activity of these enzymes in the blood of healthy animals is typically thought to result from physiologic processes.
   B. Tissue-specific enzymes come from only one cell type. Enzymes with poor tissue specificity may originate from many tissues and thus different cell types. For example, activities of enzymes commonly referred to as "liver enzymes" (e.g., ALP, AST, ALT) may increase

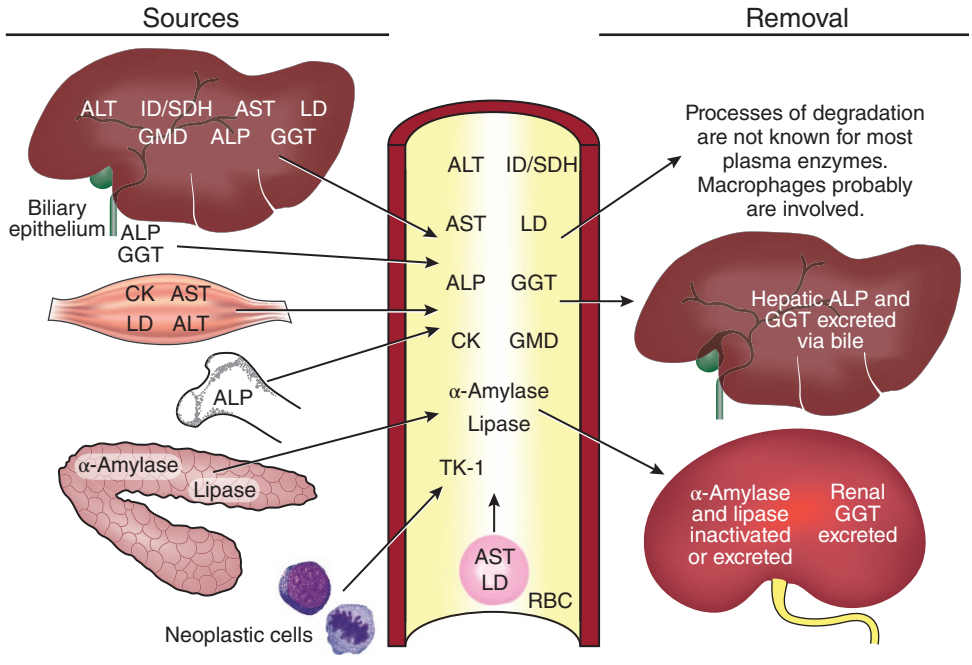


Figure 12.1. Sources and routes of removal of serum enzymes discussed in this chapter. Major sources of enzymes that contribute to increased serum or plasma activities are hepatocytes (ALT, AST, GGT, GMD, ID/SDH, LD, and ALP), biliary epithelial cells (ALP and GGT), skeletal and cardiac muscle fibers (CK, AST, LD, and ALT), osteoblasts (ALP), pancreatic acinar cells (α-amylase and lipase), and neoplastic cells (TK-1). In vitro release of AST and LD from erythrocytes can increase serum or plasma activities. Kidneys either inactivate or excrete α-amylase and lipase. The biliary system is a route of excretion of hepatic ALP and GGT. GGT released from damaged renal tubular cells is excreted in urine. Macrophages probably are involved in removal of damaged or degraded enzymes and enzyme–antiprotease complexes.

Table 12.2. Main cellular or tissue sources and half-lives of plasma and serum enzymes discussed in this chapter

Enzyme	Major mechanisms that lead to increased serum activity	Cellular sources of increased serum enzyme activity	Half-lives[a]
ALP	Induction (Ingestion)[b]	★ Hepatocytes (L-ALP; C-ALP in dogs) ★ Biliary epithelium (L-ALP) Osteoblasts (B-ALP) Mammary epithelium	~3 d (canine L-ALP and C-ALP)[1] ~6 min (canine I-ALP)[1] < 8 h (feline L-ALP)[2] ~2 min (feline I-ALP)[2]
ALT	Cell damage	★ Hepatocytes Skeletal myocytes	2–3 d (canine)[4]
α-Amylase	Cell damage Decreased renal clearance	★ Pancreatic acinar cells	~5 h (canine)[4]
AST	Cell damage	★ Hepatocytes ★ Skeletal myocytes Cardiac myocytes RBCs	~4 d (equine)[5] < 1 d (canine)[6,7] ~1 d (bovine)[8]
CK	Cell damage	★ Skeletal myocytes Cardiac myocytes Smooth muscle myocytes (minor)	~2 h (equine)[5,9] < 5 h (canine)[10,11]
GGT	Induction Cell proliferation Cell damage (Ingestion)[b]	★ Biliary epithelial cells Hepatocytes Hepatocytes (horses) (Mammary epithelium)	~3 d (equine)[12]
GMD	Cell damage	★ Hepatocytes	~14 h (bovine)[13] ~8 h (canine)[14]
ID/SDH	Cell damage	★ Hepatocytes	~4 h (canine)[15]
LD	Cell damage	★ Hepatocytes ★ Skeletal myocytes Cardiac myocytes RBCs	< 6 h (canine)[16]
Lipase	Cell damage Decreased renal clearance Cell proliferation	★ Pancreatic acinar cells Liver neoplasms Gastric mucosa	~2 h (canine)[4]
TK-1	Cell proliferation	★ Neoplastic cells Other cells	Not found

★ Major cellular sources of plasma enzyme activity
[a] Data from available but limited sources
[b] Colostrum intake in some species (see the text)

without hepatic disease, so such increases should not be automatically attributed to the liver.

C. The presence of an enzyme in a particular tissue does not necessarily mean that damage to that tissue will increase the activity of the enzyme in the blood (e.g., renal GGT).

II. Enzyme activity in plasma or serum increases when the rate an enzyme enters the blood exceeds the rate of enzyme inactivation or removal from the blood.[17] In general, there are five mechanisms by which plasma or serum enzyme activity may increase: (i) increased release from damaged cells, (ii) induction of enzyme synthesis, (iii) cell proliferation (hyperplastic or neoplastic), (iv) decreased enzyme clearance, and (v) ingestion and absorption.

A. Increased release of enzymes from damaged cells (major mechanism for cytoplasmic enzymes) (Figure 12.2)

1. Enzymes may escape from a cell because of damage caused by minor, reversible cell injury or by necrosis, a form of irreversible injury. If intracellular enzyme concentrations (cytosolic or mitochondrial) are greater than extracellular concentrations, and if the enzymes are active in blood, then enzymes from cells increase enzyme activity in plasma and serum.
2. Enzymes may be released from cells by the formation of membrane blebs that form after cell injury.[18,19] Blebs form because of cytoskeletal disruption caused by ATP depletion

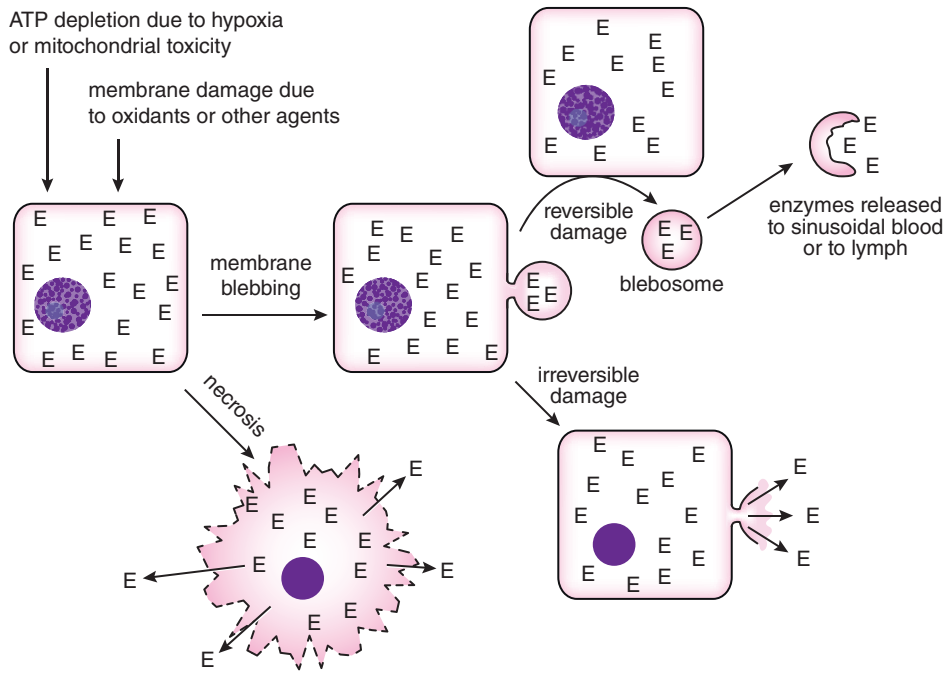


Figure 12.2. Release of cytosolic enzymes by blebbing or necrosis. Blebbing and necrosis may increase serum activity of ALT, AST, LD, ID/SDH, GMD, CK, α-amylase, lipase, and, to a mild degree, ALP and GGT. A variety of insults to cells may cause direct necrosis or membrane blebbing (irreversible or reversible damage, respectively). If irreversible cell damage occurs, cellular enzymes are released with or without bleb formation. If reversible damage occurs, blebosomes form and later lyse, thus releasing their enzymes. E, enzyme

(because of hypoxia, mitochondrial damage, or substrate depletion), toxicants binding to cytoskeletal proteins, or a variety of agents (proteases, lipases, etc.) that cause membrane damage. Once membrane blebs form, the cell may:

a. Release enzyme-containing blebosomes into extracellular fluid, where they eventually lyse and cause increased enzyme activity in plasma or serum (reversible injury)
b. Rupture at the site of bleb formation, releasing cytoplasmic enzymes into extracellular fluid (irreversible injury)
c. Repair itself without blebosome release or bleb rupture

3. Factors that affect the overall rate of enzyme loss from cells include severity of tissue damage, intracellular enzyme concentration, and intracellular enzyme location (i.e., cytoplasm, mitochondria, or cell membrane). The route of access to plasma also influences the magnitude and rate of increase.
   a. Damage to blood cells and hepatocytes rapidly increases plasma enzyme activity because these cells have close contact with plasma.
   b. Enzymes released from myocytes enter plasma via lymph and thus the rate of entry into blood is slower.
   c. Enzymes released from intestinal mucosal cells or renal epithelial cells may not enter plasma, instead entering the intestinal lumen or urine, respectively.
   d. Enzymes released from neurons may not cross the blood–brain barrier and thus may not increase plasma enzyme activity.
4. It is commonly stated that damage to cells allows enzymes to "leak" from cells because of altered cell membrane permeability. When damage is sufficient to allow an enzyme, a large protein, to leak through a porous membrane, then many other intracellular solutes (e.g., K^+ and Ca^{2+}) will leak out and the cell will die. When there is reversible cell damage that increases serum enzyme activity, this "leakage through holes" concept is not applicable unless the cells are capable of quick repair to limit the loss of contents.

B. Increased enzyme production by individual cells because of induced synthesis
   1. This is the primary mechanism for membrane-associated enzymes and may be a mechanism for mitochondrial and cytoplasmic enzymes.
   2. *Induction* refers to a stimulated increase in production of the enzyme protein via modified transcription, translation, or other processes. Endogenous substances (e.g., bile acids) or drugs such as phenobarbital, prednisolone, or prednisone may trigger induction. Increases in serum ALP activity result mostly from induction (Figure 12.3).
C. More enzyme production by a tissue because of cell proliferation, particularly for membrane-associated enzymes
   1. Cell proliferation may occur with neoplasia of the enzyme's cell of origin (e.g., increased ALP and B-ALP with osteosarcoma, or increased lipase with either pancreatic or extrapancreatic neoplasms).
   2. Cell proliferation may occur with hyperplasia of the enzyme's cell of origin (e.g., increased GGT with bile duct hyperplasia or increased B-ALP with bone growth or repair).
   3. Conversely, decreased tissue mass may be associated with decreased serum or plasma enzyme activity or concentration (e.g., trypsin-like immunoreactivity with pancreatic atrophy).
D. Enzyme removal from plasma is decreased (enzyme has an increased half-life).
   1. Some enzymes (e.g., α-amylase and lipase) are inactivated or excreted by kidneys. Decreased renal blood flow leads to decreased inactivation or excretion of α-amylase and lipase.

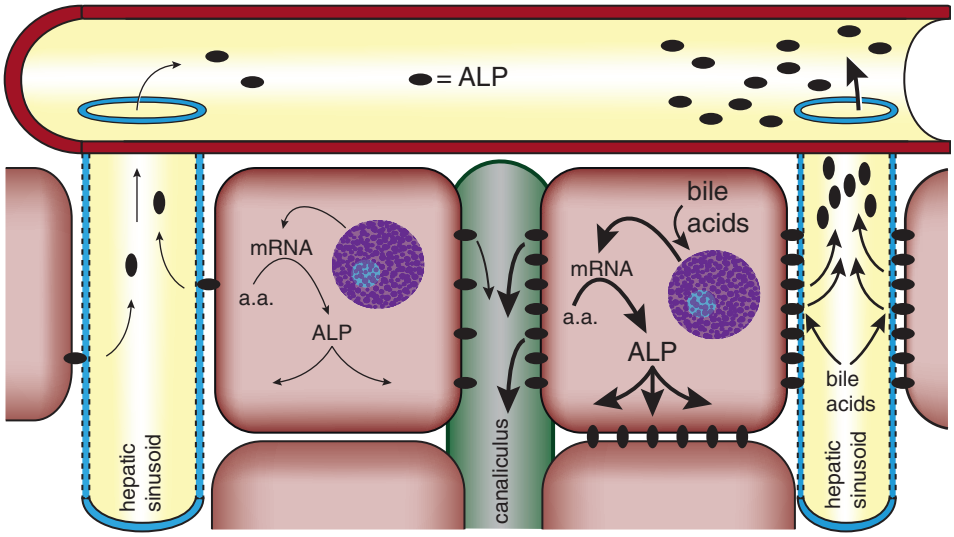


Figure 12.3. Increased production of ALP by induction. In healthy animals (*the left half of the drawing*), ALP is attached to hepatocyte membranes; more is located on the canalicular than the sinusoidal membrane. In sick animals (*the right half of the drawing*), drugs or metabolites (e.g., bile acids) induce the synthesis of more ALP that accumulates both on the canalicular and sinusoidal hepatocyte membranes. When more ALP is released from the sinusoidal membrane, in part through the actions of plasma bile acids, plasma ALP activity increases. a.a., amino acid; mRNA, messenger ribonucleic acid

2. Some enzymes form complexes with immunoglobulins or other proteins, and these so-called macroenzymes (e.g., macroamylase) may have increased survival in the circulation.
3. Other processes of enzyme inactivation include binding to circulating plasma antiproteases with subsequent uptake by macrophages or hepatocytes, and nonspecific proteolysis and then uptake by macrophages. If these processes were inhibited, removal of enzymes from plasma would be decreased.

E. Colostral enzymes (GGT and ALP) are ingested and absorbed in some species concurrently with passive transfer of immunoglobulins.

III. Decreased activity of most enzymes assessed in routine serum chemistry profiles does not have diagnostic importance. Measured enzyme activity may be reduced because of poor sample handling (enzyme degraded), the presence of an inhibitor (e.g., anticoagulants), a genetic defect in enzyme production,[20] or, potentially, a decreased mass of origin tissue (e.g., CK). Activities might appear decreased if a RI is not appropriate for the patient.

IV. Measurement of serum or plasma enzyme activities

A. Activity per unit volume (catalytic concentration)

1. Routine assays measure enzyme *activity* by detecting how fast a substrate is consumed or how fast a product forms as the enzyme catalyzes a chemical reaction.

a. Enzyme assay theory: In the presence of excess substrate (S), the reaction rate depends on the quantity of enzyme (E). More specifically, the reaction rate depends

on the rate of reaction from the enzyme–substrate complex to the product (P) + enzyme: E + S → E–S → P + E.

b. Nearly all serum enzyme assays are spectrophotometric. They may be either endpoint assays or kinetic assays:
   (1) *Endpoint assays*: The reaction is stopped at a specified time, and enzyme activity is determined from the quantity of product formed or the quantity of substrate used.
   (2) *Kinetic assays*: Multiple readings are taken during a specified time period, and enzyme activity is determined by the rate of the reaction (or rate at which product is being formed). Most current enzyme assays are kinetic assays.

2. Enzyme reactions for common clinical serum enzyme assays
   a. Figure 12.4 contains the initial chemical reactions catalyzed by the common serum enzymes. The initial reactions are usually coupled to other reactions that cause the formation or disappearance of a colored indicator that can be detected by photometry.
   b. Knowledge of the enzyme reactions promotes an understanding of enzyme nomenclature and physiologic roles of enzymes.
3. Units used to express enzyme activity and catalytic (activity) concentration
   a. U = international unit = amount of enzyme that catalyzes conversion of 1 micromole of substrate per minute under defined conditions (1 U = 1 μmol/min). In this context, substrate includes substances such as NADH or NADPH that are also called coenzymes or cosubstrates.
   b. Note that "IU" is not the abbreviation for the international unit of enzyme activity as defined by the International Union of Biochemistry and Molecular Biology. Instead, IU is the abbreviation for the mass or volume of biologically active substance considered by committee consensus to be the standard of comparison for certain pharmacologic products (e.g., some vitamins and hormones).
   c. In clinical medicine, enzyme activity is usually expressed as a catalytic (activity) concentration in U/L. However, enzyme activity concentrations are more commonly referred to as "activities" (just the U portion of U/L) than as the activity concentrations that they are. To conform to common usage, enzyme values will be referred to throughout this book as "activities" rather than as "activity concentrations" or "concentrations."
   d. Unit conversion
      1. SI unit = 1 katal = 1 mol/s
      2. 1 U = 16.67 nanokatal (SI unit rarely used in medical laboratories)
   e. Arbitrary units: A variety of historical enzyme units named after people who developed the assays may be encountered in the literature. Examples include Somogyi units (α-amylase), Sigma–Frankel and Karman units (ALT and AST assays), Rose–Byler units (lipase), and King–Armstrong and Bodansky units (ALP). Values expressed in arbitrary units may be much different from values expressed in international units, because arbitrary units may be defined by markedly different assay conditions. It is difficult to impossible to convert the arbitrary units to international units accurately.
4. The international unit (U) does not normalize methods.
   a. Because of variations in assay methods, enzyme activities measured by two different assays may be markedly different for the same sample. Table 1.10 illustrates the differences that can occur when one sample is analyzed by different methods. Thus,

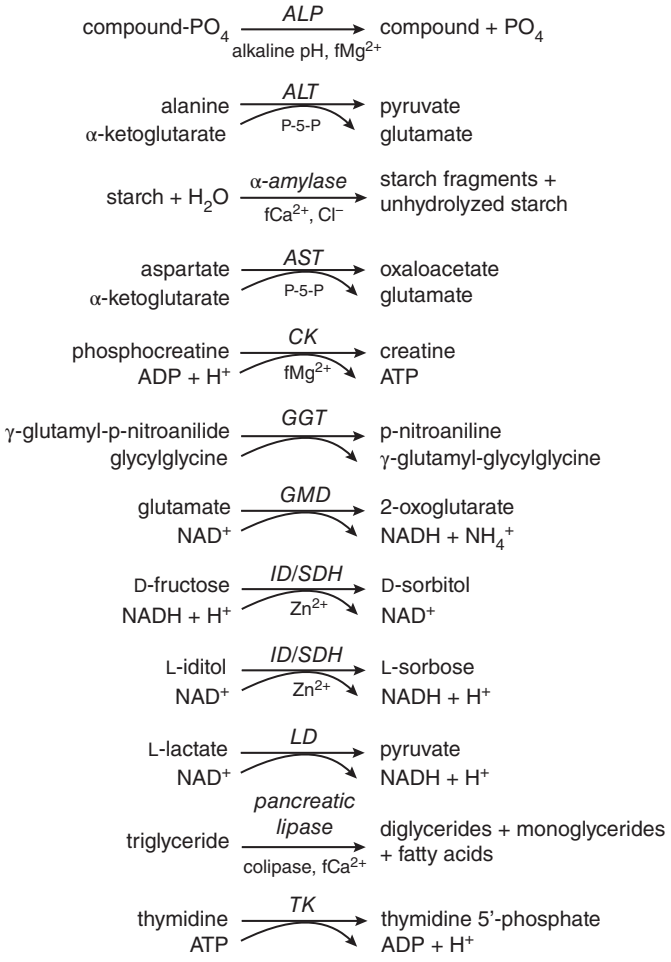


Figure 12.4. Initial reactions in assays for the serum enzymes discussed in this chapter. Assays are designed so that the rate-limiting factor is the catalytic activity of a serum enzyme. Methods of monitoring the chemical reactions that are catalyzed by the enzymes vary but typically involve absorption or reflectance photometry. ID/SDH activity is measured by using D-fructose as a substrate, but L-iditol is also a substrate and the basis of its recommended name. LD activity can be assessed in reactions that produce pyruvate from lactate or lactate from pyruvate.
ADP, adenosine diphosphate; fCa^{2+}, free calcium ion; fMg^{2+}, free magnesium ion; PO_4, phosphate; NH_4^+, ammonium

accurate interpretation of serum enzyme activity requires that patient values be compared with RIs established for the same assay system.

b. Method variations that cause different measured values include differences in:
   (1) Reactions (forward and reverse) and reagent concentrations
   (2) Substrates (especially α-amylase, lipase, and ALP assays)
   (3) pH of assay reactions (especially ALP assays)
   (4) Incubation or reaction temperatures (25 °C, 30 °C, or 37 °C)

Table 12.3. Approximate changes in enzyme activities if the same sample is analyzed at different assay temperatures

	Relative enzyme activity at:			
	25 °C	30 °C	32 °C	37 °C
Common enzymes[a]	60–80 %	100 %	110–125 %	130–210 %

[a] Including ALP, CK, LD, ID/SDH, ALT, and AST

Note: Changes are based on reported conversion factors.[21] In 2002, the International Federation of Clinical Chemistry published serum enzyme reference methods using a 37 °C reaction temperature. Previous reference methods were designed for 30 °C.[22]

(5) Use of cofactors (e.g., P-5-P)
(6) Use of activators (e.g., N-acetylcysteine for CK)
(7) Use of inhibitors (e.g., adenosine monophosphate and diadenosine pentaphosphate to inhibit adenylate kinase for CK assays)
(8) Measurement times and calculations

c. Most serum enzymes used diagnostically have near maximal activity at normal body temperatures (37–39 °C). Table 12.3 shows the relative enzyme activity at different assay temperatures. For some enzymes, enzyme activity measured at 37 °C may be three times as great as the activity measured at 25 °C in a given system. Unstable or undesired instrument assay temperatures may contribute to assay variability.

B. Mass per unit volume
1. Enzymes are proteins, and immunologic assays have been developed to measure their concentration in terms of mass per unit volume.
2. The concentrations of PLI and trypsin-like immunoreactivity (see Chapter 15) are reported as µg/L. The concentrations of these proteins are small compared to those of other common serum protein analytes such as albumin or fibrinogen (commonly reported in g/dL and mg/dL, respectively).
3. Proteins detected by immunologic assays for enzymes may include active forms and inactive or precursor forms.

V. Enzyme nomenclature
A. The recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology are used for naming enzymes (iubmb.qmul.ac.uk/enzyme).[23] Most enzymes were named by the reaction substrate first (e.g., alanine and creatine) followed by one of six types (1–6) of reaction the enzyme catalyzes: (1) oxidoreductases, including hydrogenases, oxidize or reduce, (2) transferases (e.g., transaminases transfer amino groups and kinases transfer phosphate groups), (3) hydrolases, (4) lyases, (5) isomerases, and (6) ligases. Each enzyme is also identified by a unique EC number that describes the specific reaction the enzyme catalyzes (e.g., EC 1.1.1.14 for iditol dehydrogenase), the first digit representing one of the six major enzyme types – an oxidoreductase (EC 1) in the case of ID/SDH.
B. In the conventional system, there was not a systematic method of naming enzymes. Conventional names are still used by some laboratories and authors.
C. Enzyme abbreviations

1. An international system for abbreviations has not been adopted. Abbreviations in this textbook were recommended by a group of chemists in 1975.[24]
2. For the conventional system, most abbreviations are "hand-me-downs" from one "school" or another.

VI. Sample processing, handling, and storage for common clinical serum enzymes

A. Sample type

1. Serum or heparinized plasma may be used; it is preferable for dogs and cats to be fasted. Laboratories should specify whether their RIs are for serum, plasma, or either. Harvest serum within 1 h of blood collection but allow time for clot formation and retraction (see Chapter 1, Samples, Section I.D). Prolonged contact with the clot will allow some enzymes to escape from RBCs (e.g., AST and LD).
2. Plasma can be used for some enzymes but not others.
   a. Most anticoagulants tie up divalent cations (Ca^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+}), and some enzymes require divalent cations as cofactors for them to have catalytic activity (Mg^{2+} for ALP and CK, Ca^{2+} for lipase, and Zn^{2+} for ID/SDH).
   b. Compared to serum, heparinized canine plasma was reported to have enough greater CK and LD activity and enough lower GGT activity to impact some diagnostic conclusions.[25]
   c. In rat samples, both heparinized plasma and serum with added heparin had greater GGT activity than did serum alone.[26] Such interference data for current GGT assays in domestic species were not found.

B. Sample handling

1. Enzyme activity depends on a protein's conformation. Changes in conformation may alter cofactor or catalytic sites and thus change activity.
2. There is considerable variation in published data regarding enzyme stability in serum or plasma. For a general guideline, most enzymes are stable for 24 h in serum at 24 °C and 4 °C. The degree of deterioration that occurs after 24 h (at 24 °C, 4 °C, −20 °C, or −70 °C) varies among enzymes and in some cases among species. Rate of deterioration might be different in certain pathologic states (e.g., inflammation) compared to health.
3. Specifics regarding enzyme stability and handling are provided in specific enzyme sections of this chapter. It is desirable to follow best practices.

C. Sample quality

1. Hemolysis, icterus, or lipemia can cause errors in measurement of many analytes, including enzymes, due to interference with light transmission (Chapter 1, Quality Of Laboratory Results, Section I.C.6). Hemolysis can also interfere with measurement of enzymes in serum or plasma for other reasons:
   a. Enzymes of RBCs may be added to serum or plasma, thus increasing serum or plasma activities (e.g., AST, LD, and possibly ALT).
   b. Chemical constituents of RBCs may participate in the enzyme assay, e.g., glucose-6-phosphate, ATP, or adenylate cyclase for CK activity.
2. Interference due to hemolysis, lipemia, icterus, and other factors may vary with the assay method, so assay specifics should be assessed via package inserts or laboratory personnel when questions about interferences arise.
3. Other factors that may reduce sample quality and interfere with enzyme activity include excess heparin and contamination with ethylenediaminetetraacetic acid (EDTA).

VII. Interpretation of increased serum or plasma enzyme activities

A. Values for enzyme activities may vary considerably among assay methods, but the degree of increase above the URL should be similar. Therefore, if an enzyme's activity is increased, the degree of increase is usually determined by dividing the patient's enzyme value by the URL, e.g., if a patient's ALT activity = 500 U/L with a RI of 10–50 U/L, then the ALT value is 10 times the URL (10 × URL).

B. The magnitude of increased enzyme activity may limit the possible explanations. For example, an ALT activity of 15 × URL is probably caused by hepatocyte damage, not severe muscle damage. Similarly, an ALP activity of 10 × URL is typically considered too great for B-ALP activity in adult dogs, but the ALP activity could be due to either L-ALP or C-ALP.

C. Consider the half-life of the serum enzymes. For example, CK has a shorter half-life than AST. Thus, after a single insult to muscle, CK activity is expected to return to the RI sooner than AST activity.

D. Integrate potential pathologic processes with other patient information, including physiologic variation (e.g., age, stage of pregnancy or postpartum period), to form ideas or explanations for an animal's illness. Although several minor breed, gender, seasonal, lactational, and age-related differences have been reported for enzymes, only those that are likely to affect clinical interpretations will be noted in the text.

VIII. Significance of increased serum enzyme activities

A. Serum enzyme activities are markers or indicators of pathologic processes (e.g., hepatocyte injury or cholestasis) and not of specific diseases. Many types of diseases may cause similar pathologic processes and enzymatic changes.

B. For the cytoplasmic enzymes (ALT, α-amylase, AST, CK, ID/SDH, LD, and lipases), the magnitude of increase may relate to the severity of damage, i.e., slight damage may cause values < 2 × URL, whereas severe damage might cause values > 50 × URL. However, magnitude of increase does not differentiate reversible damage from irreversible damage, or focal damage from diffuse damage.

C. A mild increase (e.g., < 3 × URL) might not be very important in one animal because other findings clearly indicate a definite diagnosis. However, such mild changes often reflect disease and might provide the only clue of active disease when other laboratory changes are mild or absent and clinical findings are vague.

D. Because of the method of characterizing a patient's enzyme activity (i.e., comparing it to the URL), the magnitude of increase is not accurately described for most animals. For example, if a dog's ALT activity prior to disease was 20 U/L and it rose to 200 U/L after the onset of disease, then it rose to 10 times the baseline value. However, if the ALT RI was 20–70 U/L, then the patient's 200 U/L value would represent an increase of only about 3 × URL and might be considered a mild increase. Because baseline values reflecting health are often not available for individual animals, we must consider variations among animals when interpreting serum enzyme activity.

E. Enzyme activity from a single sampling might not reflect the dynamic changes that may be occurring in a patient (Figure 12.5). Sampling again in 2–3 d may provide a truer image of the pathologic processes.

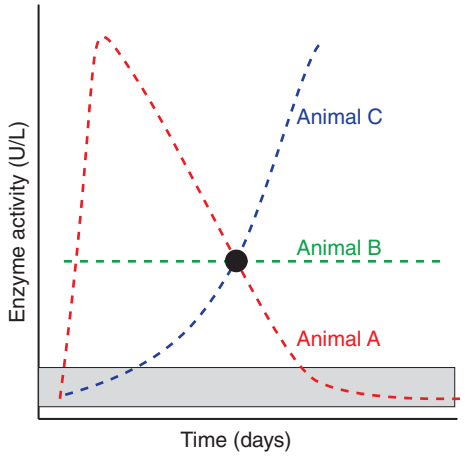


Figure 12.5. Interpretation of a single enzyme activity. On the day of sampling, all three patients' enzyme activities (●) were about 3 × URL (*shaded area is the RI*). For animal A (- - -), the 3× value occurred during the disappearance phase after an acute increase. For animal B (- - -), the 3× value reflects a persistent pathologic process. For animal C (- - -), the 3× value occurred during a progressive pathologic process. Thus, the clinical significance of the single measurement of enzyme activity can vary from animal to animal.

ALANINE TRANSAMINASE (ALT) (SYNONYM ABBREVIATION: GPT)

I. Physiologic processes, concepts, and facts
   A. *ALT* (EC 2.6.1.2) is a cytoplasmic enzyme that catalyzes a reversible reaction that converts alanine to pyruvate (Figure 12.4), which can enter the gluconeogenesis pathway or the Krebs cycle.
   B. Two ALT isoenzymes, ALT1 and ALT2, have been characterized in people and rodents, and ALT1 has been cloned in dogs, but clinical enzymatic assays do not differentiate them.[27,28]
   C. Inter-individual variation in feline serum ALT activity may be great enough to warrant interpretation of patient ALT in comparison to individual baseline values rather than to population-based RIs.[29,30] This may or may not be true for other domestic mammals.

II. Tissue sources of increased serum or plasma ALT activity and ALT half-life are listed in Table 12.2.

III. Stability
   A. ALT stability varies among species,[31] with ALT in serum of some species being susceptible to deterioration during freezing and thawing; it may lose 60 % of original activity.[32] In a study involving bovine samples, ALT was stable in heparinized plasma for 6 wk at −20 °C but was not stable overnight in frozen serum; ALT was stable at 20 °C for 2 and 4 d in serum and plasma, respectively.[33]
   B. ALT was stable in canine serum and heparinized plasma samples for 3 d when stored for 1 d at 4 °C followed by 2 d at 20 °C (mimicking conditions for some mailed samples).[25]

IV. Analytical concepts
  A. The major factor affecting serum or plasma ALT activity among assay systems is the presence or absence of the cofactor P-5-P.
    1. Conversion of apoenzymes to holoenzymes with cofactor addition is recommended, although the effect is variable and often not of diagnostic concern.
    2. In dogs and cats, the use of P-5-P was associated with a median increased ALT activity of about 10 % (–27 % to 114 %), but, in rare cases, markedly increased activity (336 % and > 14,000 %) occurred with the addition of P-5-P.[34,35] In horses, addition of P-5-P yields mildly to moderately greater mean serum ALT activities;[35,36] however, ALT is typically not assessed in horses (see VI. Species Differences, below).

V. Increased ALT activity (Table 12.4)
  A. Hepatocyte damage (reversible or irreversible) may occur because of a variety of insults (infectious or noninfectious causes of inflammation, hypoxia, toxicants, trauma, etc.). Increases in canine ALT activity that are associated with glucocorticoid therapy in some dogs[37,38] appear to be caused by glucocorticoid-induced hepatopathy instead of direct enzyme induction as is sometimes stated.[39] Glucocorticoids did not increase ALT synthesis in cultured hepatocytes.[40]
  B. ALT activity may be increased in dogs treated with phenobarbital. In a study involving 12 phenobarbital-treated dogs, data from the histologic examination of liver and chemical

Table 12.4. Disorders or conditions that cause increased ALT activity

Hepatocyte damage (dogs and cats)*
Degenerative:* hypoxia caused by anemia or congestion
Anomalous: portosystemic shunt (typically mild)
Metabolic:* lipidosis, diabetes mellitus, feline hyperthyroidism, steroid hepatopathy (endogenous steroid production)
Neoplastic:* lymphoma, metastatic neoplasms in liver, hepatocellular carcinoma
Nutritional: copper toxicosis, hemochromatosis
Inflammatory*
Infectious: leptospirosis, histoplasmosis, feline infectious peritonitis, bacterial cholangiohepatitis, infectious canine hepatitis
Noninfectious: chronic hepatitis, cirrhosis
Inherited: copper storage disease, lysosomal storage diseases
Toxic:* steroid hepatopathy (exogenous steroid source), anesthetic agents, xylitol, tetracycline, carprofen, phenobarbital, lomustine, amatoxins (certain mushrooms), cyanobacterial toxins (microcystin), aflatoxins, cycad (sago) palm ingestion
Traumatic:* hit by car
Skeletal muscle damage (mild relative to CK changes)
Inherited: canine muscular dystrophy
Traumatic: hit by car, gunshot

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders and conditions are not complete but are provided to give examples. Some conditions could fit into multiple DAMNIT categories.

analysis of liver homogenates indicated that induction did not cause the increased ALT activity.[41] Increased ALT has also been reported in a minority of phenobarbital-treated cats, but the association is less clear.[42]

C. ALT might be released from hepatocytes during reparative stages of hepatic disease, but this would be impossible to differentiate clinically from the underlying injury.

D. Spontaneous muscle damage typically is not associated with substantial increases in ALT activity, but increased ALT activity does occur with some muscle diseases in dogs and cats, and it can be marked.

1. Young dogs with muscular dystrophy may have markedly increased serum CK activity (> 500 × URL) and have mild to moderate increases in serum ALT activity (< 7 × URL).[43,44] Some dystrophic dogs occasionally have extreme ALT increases (values 20 times as great as those in nondystrophic dogs in the same colony).
2. Dystrophin-deficient cats with acute rhabdomyolysis had markedly increased CK activity (89–2000 × URL) and increased ALT activity (6–19 × URL).[45]

E. Depending on the degree of hemolysis and the assay, in vitro hemolysis might cause a mild to moderate false increase in ALT activity. In a study using canine serum, marked hemolysis had no effect in some assays and a moderate effect in others.[46]

VI. Species differences

A. In dogs and cats, ALT is a major marker of hepatocyte damage, but its serum or plasma activity is also increased by severe muscle disease.

B. Hepatocytes of horses and cattle have so little ALT that it is not a useful marker of hepatocyte damage in these species. ALT may increase with substantial hepatocellular damage in horses, but it more reliably increases with muscle damage.[17,47]

C. Information was not found regarding utility of ALT as a marker of hepatocellular damage in camelids.[48]

ASPARTATE TRANSAMINASE (AST) (SYNONYM ABBREVIATION: GOT)

I. Physiologic processes, concepts, and facts: *AST* (EC 2.6.1.1) is a cytoplasmic and mitochondrial enzyme that catalyzes the reversible reaction converting aspartate to oxaloacetate (Figure 12.4), which can enter the Krebs cycle.

II. Tissue sources of increased serum or plasma AST activity and AST half-life are listed in Table 12.2.

III. AST was stable for 3 d in canine serum and heparinized plasma samples stored at 4 °C for the first day and at 20 °C for the next 2 d.[25]

IV. Analytical concepts: As for ALT, assay methods including the cofactor P-5-P may generate different results from those lacking P-5-P.

V. Increased serum or plasma AST activity (Table 12.5)

A. Hepatocyte damage (reversible or irreversible) may occur because of a variety of insults (infectious or noninfectious causes of inflammation, hypoxia, toxicants, trauma, etc.).

B. Evidence in rats suggests that increased synthesis by reactive or regenerative hepatocytes may contribute to serum AST increases after hepatic injury, but this would be impossible to differentiate clinically from the underlying injury.

Table 12.5. Disorders or conditions that cause increased AST and LD activity
Hepatocyte damage*
Dogs and cats (see hepatocyte damage conditions listed for increased ALT in Table 12.4)*
Horses and cattle*
Degenerative:* hypoxia caused by anemia or congestion; cholelithiasis
Metabolic:* lipidosis or fat cow syndrome, diabetes mellitus, equine hyperlipidemia
Neoplastic: lymphoma, metastatic neoplasia, hepatocellular carcinoma
Inflammatory*
Infectious: bacterial hepatitis (Tyzzer's disease), bacterial cholangiohepatitis, infectious necrotic hepatitis, hepatic abscess, equine parvoviral hepatitis (likely the same as serum hepatitis or Theiler's disease)
Noninfectious: chronic hepatitis, cirrhosis
Toxic: iron toxicity, pyrrolizidine alkaloid–containing plants, aflatoxins, alsike clover toxicity, cyanotoxicosis (from cyanobacteria), copper toxicity
Skeletal or cardiac muscle damage (see muscle damage conditions listed for increased CK in Table 12.8)*
Leakage from RBCs
In vitro hemolysis or prolonged RBC contact with serum or plasma*
In vivo hemolysis

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders and conditions are not complete but are provided to give examples. Some conditions could fit into multiple DAMNIT categories.

C. Myocyte damage (reversible or irreversible) may be caused by a variety of insults (e.g., trauma, infection, hypoxia). Dogs with experimentally induced hypothyroidism developed mildly increased AST activities (mean about 2 × URL) due to a myopathy.[49]
D. Hemolysis (intravascular or in vitro), delayed removal of serum from clot, or delayed separation of plasma from cells may cause mild to moderate increases.

VI. Species differences
A. In horses and cattle, AST is a common marker of hepatocyte damage, but muscle damage and hemolysis also increase serum or plasma AST activity.
B. AST is recognized as a useful marker of hepatocellular injury in camelids. Muscle damage also increases AST activity in these species.[48]
C. AST can also be used as an indicator of hepatocyte or muscle damage in dogs and cats, but its diagnostic utility is limited because it is less tissue-specific than ALT in dogs and cats.[17] AST is reported to have a shorter half-life than canine ALT (< 1 d compared to 2–3 d),[3,6] so it may be less sensitive than ALT as a marker of active hepatocyte or myocyte damage.

L-LACTATE DEHYDROGENASE (LD) (ALSO ABBREVIATED LDH)

I. Physiologic processes, concepts, and facts
A. *LD* (EC 1.1.1.27) is a cytoplasmic enzyme that catalyzes a reversible reaction that converts pyruvate to L-lactate (Figure 12.4).

B. *LD isoenzymes* are tetramers composed of 0–4 heart subunits (H) and 0–4 muscle subunits (M). In principle, identification of the five different LD isoenzymes could increase the diagnostic value of LD activity in serum or plasma. However, LD isoenzyme analysis is not widely available.

II. Major tissue sources of increased serum LD activity and LD half-life are listed in Table 12.2.

III. Stability: LD was stable in serum and heparinized plasma stored for 1 d at 4 °C followed by 2 d at 20 °C.[25] In another study, LD activity was stable for 4 wk in canine serum stored at −20 °C.[50] However, these studies assessed healthy dogs, and LD isoenzymes have different temperature sensitivities, so stability of total LD activity may vary with disease and isoenzyme profiles.[50]

IV. Analytical concepts: Some assays measure LD activity in a pyruvate-to-lactate reaction, others in a lactate-to-pyruvate reaction. The LD activity in the two types of reactions can vary considerably,[51] perhaps because LD-1 is inhibited by high pyruvate concentrations and LD-5 maintains activity at high pyruvate concentrations.[52] Significant differences can also occur with assay variations using the same reaction, and differences might vary with the species and disease because of differences in isoenzyme predominance.[53]

V. Increased serum or plasma LD activity (Table 12.5)
   A. In all species, LD is a marker of hepatocyte damage, but LD activity is also increased by muscle damage and hemolysis (even mild). Dogs with experimentally induced hypothyroidism developed a myopathy with mildly increased LD activities (mean < 2 × URL) and increases in the LD-5 isoenzyme.[49]
   B. Increased LD activity may be caused by reversible or irreversible, focal or diffuse cell damage.
   C. By itself, LD activity is a screening test for hepatocyte or muscle damage. In a group of tests, an increase in LD activity provides further evidence to support changes in activities of enzymes that are more tissue specific (i.e., ALT, ID/SDH, and CK).
   D. Increased LD activity or changes in LD isoenzyme profiles attributed to neoplasms have been reported in dogs with lymphoma and mammary malignancies.[54,55] However, the source of LD in these dogs, whether from the neoplastic cells or others, is unclear.

L-IDITOL 2-DEHYDROGENASE (ID) (SYNONYM: SORBITOL DEHYDROGENASE, SDH)

I. Physiologic processes, concepts, and facts
   A. *ID* (EC 1.1.1.14) is a cytoplasmic enzyme that catalyzes a reversible reaction in the conversion of glucose to fructose through the sugar alcohol intermediate D-sorbitol (D-glucitol).
   B. The established name for the enzyme is L-iditol 2-dehydrogenase, as it catalyzes the conversion of L-iditol to L-sorbose, but iditol is not a substrate or product in clinical assays. The enzyme is more frequently referred to as *sorbitol dehydrogenase* (SDH). In the clinical assay, D-fructose is the substrate and D-sorbitol is the product.

II. Tissue sources of increased serum ID/SDH activity and ID/SDH half-life are listed in Table 12.2.

III. Stability: ID/SDH is stable (> 90 % activity remains) in equine serum for up to 5 h at 21 °C, 24 h at 4 °C, and 48 h at –30 °C; 73 % of ID/SDH activity was lost by 24 h at 21 °C and 28 % was lost by 72 h at 4 °C.[56] Stability was acceptable (± 20 % of baseline) in promptly harvested equine heparinized plasma stored for 4 h at 18–25 °C, 2–6 °C, and –18 to –22 °C, and also for 24 h at 2–6 °C; however, individual samples had greater increases or decreases in ID/SDH activity with longer storage (24 or 72 h) at these temperatures.[57] The impact of assay imprecision was not assessed.

IV. Analytical concepts: ID/SDH assays are not consistently included in serum chemical profiles, in part because the enzyme's stability is a concern for routine handling of mail-in samples, and in part because of reagent stability and availability.

V. Increased serum or plasma ID/SDH activity
   A. This indicates hepatocyte damage that may be reversible or irreversible, focal or diffuse. It is unusual to find ID/SDH activity > 10 × URL.
   B. ID/SDH activity is used primarily in horses, cattle, and camelids because other common hepatic cytosolic enzymes (AST and LD) are not liver specific, and ALT is not a useful marker of hepatocyte damage in those species.
   C. ID/SDH activity is sometimes used as a marker of hepatocyte damage in dogs and cats, but ALT assays are more commonly available, and ALT is more stable.

GLUTAMATE DEHYDROGENASE (GMD) (ALSO ABBREVIATED GD, GDH, GLD, and GLDH)

I. Physiologic processes, concepts, and facts
   A. *GMD* (EC 1.4.1.2) is a mitochondrial enzyme[17] that catalyzes a reversible reaction converting glutamate to 2-oxoglutarate. In people and rats, centrilobular hepatocytes have relatively more GMD activity than periportal hepatocytes.
   B. GMD is not a common abbreviation for this enzyme, but it is a recommended abbreviation.[24] Two more common abbreviations are GDH and GLDH. GDH is also used as an abbreviation for glycerate dehydrogenase and glucose dehydrogenase.

II. Tissue sources of increased serum GMD activity and GMD half-life (Table 12.2): Serum and plasma GMD activity is mostly from hepatocytes, but GMD is also found in many other tissues (e.g., kidney, intestine, and muscle). However, damage to those tissues does not increase serum GMD activity because the enzyme does not enter plasma (e.g., kidney and intestine) or there is too little in the tissues (e.g., muscle and salivary glands).

III. Stability: In a study of samples from eight dogs, GMD was reportedly stable for 48 h at 20 °C, for 1 wk at 4 °C, and for at least 6 mo at –20 °C. However, some individual samples appeared less stable.[58] GMD was also stable in serum or heparinized plasma from healthy dogs when samples were stored at 4 °C for 1 d followed by 20 °C for 2 d.[25]

IV. Analytical concepts: Commercial assays for GMD were not available in the United States until relatively recently, so their evaluation and application have been limited in this country.

V. Increased GMD activity

A. This typically is thought to indicate irreversible hepatocyte damage that may be focal or diffuse.[19] However, it is unclear if mitochondrial damage combined with reversible cytoplasmic blebbing could also cause GMD increases. Disorders or conditions (listed in Tables 12.4 and 12.5) that cause hepatocyte damage may increase serum GMD activity.
B. GMD activity appears to be a sensitive indicator of hepatocellular disease in dogs.[59]
C. Some dogs treated with prednisone[58] or phenobarbital[60] had increased serum GMD activity, but it is not known whether the increase was caused by hepatocyte damage or direct drug induction.
D. Based on results of liver biopsies, the diagnostic sensitivity of increased GMD activity for differentiating the presence of hepatic disease from the absence of hepatic disease in horses was not as good as that of GGT activity, but GMD had a better positive predictive value.[61] However, this may vary with the types of hepatic disease encountered. Accuracy of diagnosing subclinical sporidesmin toxicity (facial eczema) was greater when using combined GMD and GGT results compared to using either test alone.[62]
E. Increased serum GMD has been reported in cattle with hepatic lipidosis and other conditions (toxic, infectious, neoplastic, metabolic) that cause hepatocellular damage.

ALKALINE PHOSPHATASE (ALP)

I. Physiologic processes, concepts, and facts
A. *ALP* (EC 3.1.3.1) enzymes hydrolyze phosphate monoesters in an alkaline environment.
B. Many cell membranes have ALP activity, but only a few produce enough ALP to increase serum or plasma ALP activity. Physiologic functions of ALP are diverse. L-ALP may be involved in the degradation of endotoxins,[63] I-ALP functions include inactivation of bacterial endotoxin and other pathogen-associated molecular patterns,[64,65] and B-ALP is involved in the mineralization of bone.[66]
C. Inter-individual variation in feline serum ALP activity appears to be great enough to warrant interpretation of patient ALP in comparison to individual baseline (subject-based) values rather than to population-based RIs.[29,30] This may or may not be true for other domestic mammals.
D. Adequate ingestion of colostrum increases serum ALP activity in some species.
1. Serum ALP activity in calves increases up to three times presuckling values 1–2 d after nursing. At least part of this increase is caused by the ingestion of colostrum. The ALP activity nearly returned to presuckling values by day 3.[67]
2. Total serum ALP activity does not increase after colostrum ingestion by foals.[68]
3. Serum ALP activity may be up to 30 × URL in postsuckling pups; the increased ALP activity might be due to colostral ALP or increased ALP production induced by ingestion of colostrum.[69] Serum ALP activity returned to presuckling values within 10 d.
E. Increased ALP activity (total and B-ALP) associated with bone growth in juveniles
1. Puppies have greater total serum ALP activity than adult dogs, particularly until 6 mo of age.[70,71] Total ALP activity drops to the adult plateau at approximately 1 yr of age.[71,72] Increased ALP activity in puppies (compared to adult values) is due to B-ALP once postcolostral ALP has cleared.[73]
2. Serum ALP activity in presuckling foals can be 20 × URL for healthy adult horses but decreases to about 5 × URL by 3 wk of age.[68] The increase in total ALP activity is due to B-ALP.[68]

3. Serum B-ALP activity and age were negatively correlated in dairy cattle ranging in age from 11 d to 10 yr.[74]
4. Crias have greater serum ALP activity than adult alpacas.[75]

F. Compared to precalving values, plasma B-ALP activity of dairy cows may be mildly decreased 1–2 wk after calving, and periparturient B-ALP values are lower in multiparous compared to primiparous cows.[74,76]

II. Tissue sources of increased serum ALP activity and ALP half-life (Table 12.2)

A. In domestic mammals, there appear to be two ALP genes, each producing one ALP isoenzyme that undergoes posttranslational modifications to yield different isoforms in different cell types (Figure 12.6). A rare mutation in the tissue-nonspecific ALP gene caused severe skeletal disease in affected Karelian bear dogs, and serum ALP activity was decreased in the affected puppy that was tested.[20]

B. *C-ALP* is a unique enzyme in canids that is produced by hepatocytes when stimulated by glucocorticoids. Although its amino acid sequence is the same as I-ALP, it is more highly glycosylated, has a much longer half-life, and can cause marked increases in serum ALP activity. In contrast, I-ALP has not been shown to increase serum ALP activity in domestic mammals.[77] Substantial increases in serum ALP activity with a predominance of I-ALP occurs rarely in asymptomatic people with mutations in the I-ALP gene.

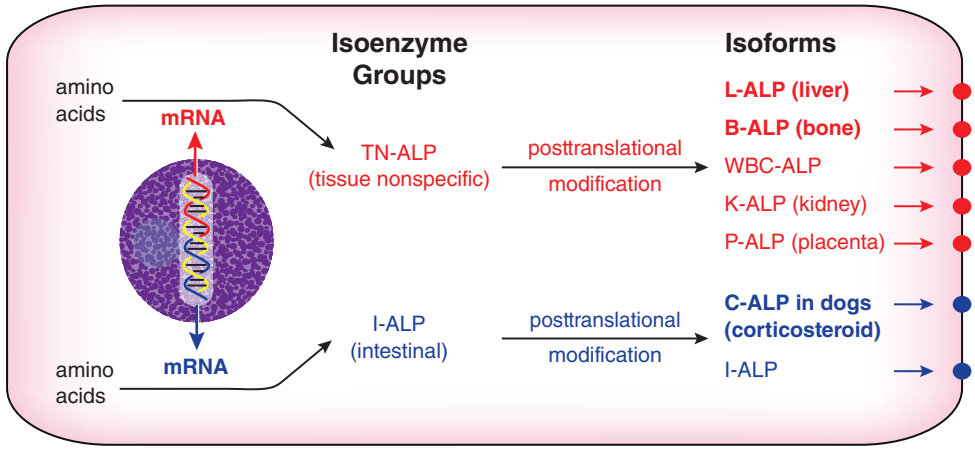


Figure 12.6. Isoenzymes and recognized isoforms of ALP. Isoenzymes (also called isozymes) catalyze the same reaction but are encoded by different genes that yield distinct polypeptides from the assembly of different sequences of amino acids. In domestic mammals, there are two ALP genes yielding TN-ALP and I-ALP isoenzyme groups. Different isoforms of these two polypeptides are created by different posttranslational modifications (e.g., glycosylation) occurring in different cells and tissues. Each ALP isoform is incorporated into the cell membrane of its cell of origin. The ALP isoforms responsible for most increases in plasma ALP activity are shown in bold. L-ALP originates from hepatocytes and biliary epithelium. B-ALP originates from osteoblasts. C-ALP, in canids only, originates from hepatocytes when they are stimulated by corticosteroids. P-ALP (from placental trophoblasts) may contribute to increased plasma ALP activity in late-term cats. WBC-ALP (in white blood cells), I-ALP (in enterocytes), and K-ALP (from tubular epithelium in the kidney) have not been shown to contribute to increased serum or plasma ALP activity.

III. Stability: ALP in serum and heparinized plasma from healthy dogs was stable for 3 d when samples were stored 1 d at 4 °C followed by 2 d at 20 °C.[25]

IV. Analytical concepts
- A. In routine assays, measured ALP activity represents total ALP activity and typically includes activity from L-ALP and B-ALP (also C-ALP in dogs). The relative contributions to total ALP activity vary with age. In healthy dogs, C-ALP contributes about 10–30 % of total ALP activity; it contributes more in older dogs than in younger dogs.[78]
- B. Although usually not measured separately, it is helpful to consider the different isoforms of ALP when interpreting ALP activity.
- C. The relative contributions of the different ALP isoforms to total ALP activity can be determined by enzyme electrophoresis, selective inhibition, thermal stability, or selective precipitation.[73,79–81]
  1. Affinity electrophoresis is the conventional or standard method but requires special equipment and is time and labor intensive.[82] L-ALP, C-ALP, and B-ALP can be identified in canine serum.
  2. Levamisole selectively inhibits L-ALP and B-ALP activity, but C-ALP is relatively resistant. Measurement of serum ALP activity with and without the addition of levamisole can help determine how much of the serum ALP activity is due to C-ALP.
  3. C-ALP is relatively heat stable at 56 °C and 65 °C, whereas L-ALP and B-ALP are relatively heat labile; B-ALP is more heat labile than L-ALP. Measurement of serum ALP activity prior to and after sample heating can help determine how much of the serum ALP activity is due to C-ALP.
  4. B-ALP and C-ALP are precipitated by wheat germ lectin, whereas L-ALP is not. ALP activity before and after the precipitation can be used to estimate B-ALP activity; in dogs, this requires concurrent determination of C-ALP activity.
  5. The contribution of each ALP subtype to total ALP activity can be determined by concurrent testing (e.g., in dogs, levamisole inhibition to assess C-ALP and wheat germ lectin precipitation to assess L-ALP, with the B-ALP contribution being calculated).
- D. Increased serum glucose concentration inhibits ALP activity after glucose binds to ALP (nonenzymatic glycosylation) in human patients.[83,84] To the authors' knowledge, hyperglycemia-induced reduction in ALP activity has not been documented in spontaneous disorders of domestic mammals.

V. Increased serum or plasma ALP activity (Table 12.6)
- A. Cholestasis (intrahepatic or posthepatic)
  1. Increased ALP activity appears to be primarily caused by increased production of L-ALP by hepatocytes during obstructive cholestasis. ALP production is expected to be mildly increased in functional cholestasis (see Chapter 13) as well, although documentation was not found for domestic mammals.
  2. Many diseases lead to impaired bile flow, which leads to an accumulation of bile acids in hepatocytes. Increased bile acid concentrations stimulate L-ALP production, promote accumulation of L-ALP on sinusoidal hepatocyte membranes, and are linked to increased serum ALP activity (Figure 12.3).[81,85] Bile acids promote L-ALP release from the hepatocyte membranes by promoting activity of glycosylphosphatidylinositol phospholipase D.[86,87]
  3. After experimental bile duct ligation of dogs, serum ALP is mildly increased on day 1, peaks as high as 50 × baseline at about day 10, and remains increased for weeks.[88,89]

Table 12.6. Disorders or conditions that cause increased ALP activity (the isoform produced is in parentheses)
Ingestion of ALP-rich colostrum by calves and puppies
Bone growth in juveniles (B-ALP)*
Cholestasis, either intrahepatic or posthepatic (L-ALP)*
Degenerative:* necrosis or hepatocyte swelling that leads to impaired bile flow
Metabolic:* lipidosis, diabetes mellitus, hyperadrenocorticism, cholelithiasis
Neoplastic:* bile duct carcinoma, pancreatic carcinoma, lymphoma
Inflammatory:* periportal hepatitis, cholangitis, cholangiohepatitis, pancreatitis
Toxic: pyrrolizidine alkaloid–containing plants, alsike clover toxicity, sporidesmin toxicosis
Drugs or hormones*
Phenobarbital, phenytoin, primidone (L-ALP and C-ALP[a])*
Glucocorticoids (L-ALP and C-ALP[a]):* endogenous or exogenous
Thyroxine (L-ALP and B-ALP):* feline hyperthyroidism
Scottish terriers with progressive vacuolar hepatopathy (C-ALP)
Breed-related variations (Bernese mountain dogs, Norwegian forest cats)
Increased osteoblastic activity (B-ALP): osteosarcoma, fracture repair, rickets, hyperthyroidism
Canine mammary neoplasms, benign and malignant (L-ALP, B-ALP, and C-ALP[a])
Benign familial hyperphosphatasemia in Siberian huskies (B-ALP)
Late-term pregnancy in cats (placental-ALP)
Undifferentiated acute leukemia in a dog (B-ALP)

* A relatively common disorder, condition, or mechanism

[a] C-ALP induction occurs only in canids.

Note: Lists of specific disorders and conditions are not complete but are provided to give examples. Some conditions could fit into multiple DAMNIT categories.

4. In cholestatic disorders, other substances or factors might also induce L-ALP production, or there might be decreased biliary excretion of ALP. In the absence of cholestasis, there is too little ALP associated with hepatocytes and biliary epithelium for hepatic necrosis to cause large increases in serum or plasma ALP activity.

B. Drugs or hormones
   1. Many drugs have the potential to increase L-ALP activity. Glucocorticoids (prednisone and prednisolone), phenobarbital, and primidone are commonly reported to induce L-ALP production, but induction may be direct or secondary to other drug-induced changes. The degree to which serum or plasma L-ALP activity increases typically depends on dose and duration of compound administration. It takes hours for hepatocytes to increase L-ALP production (it may take days for clinical evidence of increased production), and increased serum ALP activity may persist for 2–4 wk after removal of the drug.[39,90]
      a. Serum ALP activity increased in 11 of 12 epileptic dogs treated with phenobarbital, but the predominant ALP fraction varied, being L-ALP in five dogs and C-ALP in six, including the five dogs with the greatest increases.[41] Histologic and chemical data from liver biopsies provided evidence of hepatic injury (cell

swelling, fibrosis) and no compelling evidence for direct induction, but induction with concurrent accelerated release was not excluded.

b. Data from hepatic tissue homogenates analyzed after 32 d of prednisone injections indicate that the steroid treatment increased L-ALP production by increasing gene transcription in hepatocytes.[91]

2. C-ALP

a. In canids, glucocorticoids (e.g., endogenous cortisol or exogenous prednisone or prednisolone) induce the production of a unique ALP isoform of I-ALP called *C-ALP* (also called *CI-ALP* for corticosteroid-induced ALP, *SI-ALP* for steroid-induced ALP, and *CAP* for corticosteroid alkaline phosphatase). The increase in serum C-ALP activity begins about a week after initiation of steroid therapy. Most experimental evidence supports that glucocorticoids upregulate a specific gene in canine hepatocytes to produce this unique enzyme.[92]

b. Measuring C-ALP activity can be a screening test for canine hyperadrenocorticism because most dogs (83–100 %) with spontaneous hyperadrenocorticism have increased serum C-ALP activity.[93] However, increased C-ALP activity is not specific for hyperadrenocorticism, because there are physiologic and therapeutic reasons for increased glucocorticoid hormones and thus increased C-ALP activity.

3. Thyroxine mediates mild and clinically insignificant increases in serum and plasma total ALP activity due to increased activities of B-ALP and, usually, L-ALP in cats with hyperthyroidism.[94]

C. Scottish terriers with or without clinical diagnoses of hepatic, pancreatic, or adrenocortical disease have greater ALP activities than age-matched dogs of other breeds, and, in contrast to control breeds, increases in ALP activity with age.[95,96]

1. Despite moderate to marked ALP increases, these dogs typically appear clinically healthy.[96,97] C-ALP is the predominant ALP isoform.

2. Affected dogs secrete excessive amounts of various adrenocortical hormones (e.g., androstenedione and progesterones) before or after exogenous ACTH stimulation and have increased urine cortisol-to-Crt ratios. Hormone secretion can be suppressed by dexamethasone, indicating appropriate feedback interactions with the adrenal glands.[98]

3. These dogs develop so-called progressive vacuolar hepatopathy,[96,99] and a predisposition to develop hepatocellular carcinomas has been suggested.[99]

D. ALP variations in clinically healthy dogs and cats

1. Bernese mountain dogs: Approximately half of 32 clinically healthy Bernese mountain dogs had serum ALP activities greater than an established (details not provided) canine RI; the URL based on these 32 dogs was 2–3× that of the established canine RI. No pathologic cause of increased ALP activity was detected, suggesting a breed-specific physiologic difference necessitating a breed-specific RI.[100]

2. Norwegian forest cats: The median serum ALP activities in 28 clinically healthy Norwegian forest cats was at least twice that of three other feline breeds, and, in contrast to those breeds, most values were greater than (up to about 5×) a published RI (details not provided) for feline ALP. This should be considered when interpreting ALP values for this breed.[101]

E. Increased osteoblastic activity

1. Bone lesions that cause increased osteoblastic activity may cause increased serum activity of B-ALP and thus increased total ALP activity. Generally, the magnitude of increase in

total ALP is mild (< 4 × URL) but has been reported to be as much as 12 times the average ALP activity in serum of two healthy dogs.[80]

2. Increased total ALP and B-ALP activities in frozen serum from dogs with osteosarcoma have been associated with reduced patient survival; the degrees of increases in ALP and B-ALP were not reported.[102] Others have found that increases in B-ALP activity in frozen serum of dogs with osteosarcoma and no metastases correlate with the size of the primary mass, and that in some dogs with metastases, B-ALP decreases with therapy and is increased terminally.[103]
3. Total ALP activity increased (about 2–3 × baseline by day 10) during fracture healing in dogs.[104] The return to baseline activity corresponded with cessation of callus formation. In contrast, dogs with nonunion fractures did not have increased ALP activity during a 2 mo evaluation.
4. Canine serum ALP activity was greater in growing puppies on a phosphorus-deficient diet than in puppies on appropriately balanced diets; isoenzymes were not assessed, but increased bone turnover was suspected to have caused increased B-ALP release.[105]
5. Cats with hyperthyroidism have increased B-ALP (and L-ALP) activity that contributes to increased total ALP activity.[94]

F. Dogs with malignant and benign mammary neoplasms may have increased serum ALP activity (typically < 8 × URL) in the absence of detectable hepatic or bone metastasis.[106,107] Myoepithelial cells may produce ALP, but no mammary isoform has been described. In one study, median total ALP, L-ALP, C-ALP, and B-ALP activities were each greater in female dogs with malignant mammary neoplasms than in clinically healthy dogs; there were no differences in activities based on the type of neoplasm or presence of osseous transformation.[108] Reports of serum ALP activity in dogs with other nonosseous neoplasms were not found.

G. Benign familial hyperphosphatasemia was reported in Siberian husky puppies; hyperphosphatasemia could be attributed to B-ALP in the affected family members that were tested.[109]

H. Placental ALP may contribute to serum ALP activity in at least late-term pregnancy of cats,[110] but ALP activity did not increase in pregnant mares,[111] and the increase in serum ALP during mid-late pregnancy in bitches is mild and appears to be of hepatic origin.[112,113]

I. An adult dog with extreme leukocytosis caused by an acute leukemia had a marked increase in serum and plasma ALP activity that was caused by B-ALP.[114] Clinical evidence of bone disease was lacking, but radiography was not done. Leukemic cells might have been a source of plasma ALP activity given that (i) they were strongly positive for ALP by cytochemical staining and (ii) cell lysis caused substantial increases in sample ALP activity.

VI. Species differences

A. Dogs

1. ALP has high diagnostic sensitivity for detecting cholestasis. ALP activity may be increased before icterus and hyperbilirubinemia appear. ALP values may range from < 2 × URL to > 20 × URL.
2. Increased ALP activity induced by glucocorticoids results from induced synthesis of L-ALP and C-ALP. ALP values may range from < 2 × URL to > 20 × URL.[39]
3. Phenobarbital, primidone, and phenytoin are described in clinical studies as either inducing the synthesis of L-ALP or causing hepatopathy-associated cholestasis that increases serum L-ALP in dogs.[115]
4. Increased ALP activity caused by increased osteoblastic activity in growing dogs (production of B-ALP) is typically mild (< 4 × URL).[116]

B. Cats
   1. ALP has poor diagnostic sensitivity for detecting cholestasis and may increase before[117] or after[118] hyperbilirubinemia is detected. In comparison to dogs, feline ALP increases are limited by a shorter half-life of L-ALP (Table 12.2). ALP values may range from < 2 × URL to > 10 × URL.
   2. In 12 (80 %) of 15 of cats with lipidosis, the ALP:GGT ratio was increased (i.e., ALP activity increased more than GGT activity).[119] Only 4 (10 %) of 39 of the cats with hepatic diseases other than lipidosis (i.e., bile duct obstruction, cholangitis, cholangiohepatitis, neoplasia, hepatic necrosis, and cirrhosis) had increased ALP:GGT ratios (magnitudes of change not reported).
   3. It has been reported that from 25 % to 75 % of untreated hyperthyroid cats have increased serum ALP activities.[120,121] ALP activities typically are < 4 × URL and return to WRI after successful treatment. In such cats, the increased ALP activity is usually caused by L-ALP and B-ALP, whereas total ALP activity in healthy mature cats is primarily due to L-ALP. Some hyperthyroid cats have increased B-ALP activity in serum or plasma without a concurrent increase in total ALP activity.[120]

C. Horses
   1. ALP has poor diagnostic sensitivity for detecting cholestasis. Horses typically are icteric before ALP activity increases. ALP values may range from < 2 × URL to > 10 × URL.
   2. There is no evidence that glucocorticoid treatments increase serum L-ALP activity in horses, nor that horses produce C-ALP.[122] In two reported cases of steroid hepatopathy in horses, ALP activity was not measured.[123,124]
   3. In horses with colic or intestinal lesions, ALP activity may increase in the peritoneal fluid predominantly because of increased granulocytic ALP activity,[125] but other data suggest that intestinal ALP may contribute (see Chapter 19).[126]

D. Cattle: ALP has moderate diagnostic sensitivity for detecting cholestasis, but cholestasis disorders are uncommon in cattle.

E. Camelids: Reports regarding the clinical utility of ALP in camelids were not found.

γ-GLUTAMYLTRANSFERASE (GGT) (SYNONYM ABBREVIATION: GGTP)

I. Physiologic processes, concepts, and facts

A. *GGT* (EC 2.3.2.2) catalyzes the transfer of glutamyl groups between peptides and is involved in glutathione reactions.

B. GGT is associated with cell membranes. Many cells have GGT activity, but biliary epithelial cells, pancreatic acinar cells, and renal tubular epithelial cells are classically considered to have the greatest activity. In some species, mammary glands also have high GGT activity. In these species, GGT is associated with mammary glandular epithelial membranes and milk membranes in milk (milk membranes form with secretion of lipid vesicles and include epithelial cell membrane proteins).[127] GGT has also been detected in hepatocyte cell membranes. Liver GGT activity is greater in horses, ruminants, and pigs than in dogs.[17]

C. Inter-individual variation in equine serum GGT activity was found in one study to be great enough to warrant interpretation of patient GGT in comparison to individual baseline (subject-based) values rather than to population-based RIs.[128] This may or may not be true for other domestic mammals.

D. Ingestion of colostrum increases GGT activity in ruminants and dogs.
   1. Colostrum of cows has high GGT activity, and the GGT molecules may be absorbed from the calf intestine after colostral intake. Postsuckling calves can have GGT activities

as high as 20 × URL (using adult RIs) and up to 16 times the presuckling values.[67,129–131] This physiologic change can be used as evidence of suckling and thus as an indication of successful transfer of passive immunity.

2. Because of high colostral GGT, serum GGT activity in 1- to 3-d-old pups is up to 100 × URL (using adult RIs) but returns to presuckle values within 10 d after suckling.[69]
3. Mare colostrum contains relatively little GGT activity, and GGT activity in plasma of neonatal foals does not increase after suckling. However, serum GGT activity in foals less than 1 mo old is about 1.5–3 times as great as the GGT activity in adult horses.[132]
4. In neonatal llama and alpaca crias, serum GGT activity was commonly considered high but was not significantly associated with serum IgG concentration.[133,134] Based on those findings, GGT is not considered a reliable marker of passive transfer in these species.
5. Data regarding GGT in feline mammary glands and postsuckle kittens were not found.

II. Tissue sources of increased serum GGT activity and GGT half-life are listed in Table 12.2.

III. Stability: GGT activity in heparinized equine plasma was stable for 1 mo at −20 °C.[135] GGT activity was stable in serum and heparinized plasma for 3 d when samples from healthy dogs were stored at 4 °C on day 1 and at 20 °C on days 2 and 3.[25]

IV. Analytical concepts: With certain GGT methods (e.g., with γ-glutamyl-p-nitroanilide as a substrate), heparinized plasma yields greater GGT activities than serum,[25,26,136] and excess heparin may falsely increase GGT activity beyond acceptable limits.[137] Falsely increased GGT activity has been attributed to sample turbidity, which may occur with heparin-induced protein precipitation when using transmission but not reflectance photometry.[51,138] Therefore, serum and plasma references intervals may not be interchangeable, and excess heparin (e.g., by incomplete filling of heparin tubes) should be avoided.

V. Increased serum or plasma GGT activity (Table 12.7)
   A. Cholestasis or biliary hyperplasia
      1. Increased hepatic and plasma bile acid concentrations are expected with cholestasis. Increased bile acids or other constituents of bile may stimulate the synthesis and release

Table 12.7. Disorders or conditions that cause increased serum or plasma GGT activity

Ingestion of GGT-rich colostrum by calves and puppies
Cholestasis or biliary hyperplasia*
For cholestasis conditions, see Table 12.6
Conditions with prominent biliary hyperplasia: pyrrolizidine alkaloid–containing plants, sporidesmin toxicity, alsike clover toxicity, *Brassica* spp. (turnip, rutabaga, rape) toxicosis, fascioliasis
Drugs or hormones
Phenobarbital, phenytoin, primidone
Glucocorticoids: endogenous or exogenous
Hepatocyte damage in horses and potentially cattle (see text)
Neoplastic cells (renal or mammary carcinomas, possibly hepatocellular carcinomas)
Bernese mountain dogs (a breed variation)
Idiopathic in racehorses

* A relatively common disorder, condition, or mechanism

of GGT from biliary epithelial or hepatocyte membranes, but mechanisms of increase are not completely established.[85] Disorders that cause cholestasis may also induce biliary hyperplasia and a resultant increase in GGT activity.

2. Experimental data from rat studies indicate that increased serum GGT activity primarily depends on the degree of hyperplasia of biliary epithelial cells and not on induction of GGT synthesis, hepatocyte damage, or cholestasis.[139]
3. In several toxicities affecting horses or ruminants, there is prominent biliary hyperplasia that appears to be the major contributor to increased serum GGT activity (Table 12.7).

B. Drugs or hormones
1. Serum GGT activities transiently increased to 2–3 × baseline values from weeks 13 to 17 in dogs given phenobarbital for 27 wk, but mean activity did not exceed the RI.[140]
2. Induction may not increase serum GGT activity in dogs being treated with glucocorticoid hormones, since glucocorticoids did not induce GGT synthesis in cultured hepatocytes.[40] However, in prednisone-treated dogs, hepatic and serum GGT activities increased proportionately, which suggested induction or the effects of steroid hepatopathy.[39]

C. Hepatocyte damage in horses and adult cattle: Experimental data in horses indicate that acute hepatocellular necrosis can cause rapid mild increases (< 4 × baseline) in GGT activity.[141] Similar results were found with experimentally induced acute hepatic necrosis in adult cattle.[8] Comparable hepatocellular insults caused little or no GGT change in calves, sheep, and dogs. The rapid increase in serum GGT activity suggests it may be the result of hepatocellular necrosis rather than delayed induction from secondary cholestasis. Of 21 horses and one donkey with iron overload, 21 had increased serum GGT activity, and all 12 with histologically assessed hepatic tissue had fibrosis, hepatitis, and iron accumulation without reported biliary hyperplasia.[142] Hyperbilirubinemia was present in only 3 of the 16 horses for which it was assessed.

D. Increased production by a neoplasm: GGT activity was approximately 19 × URL in a dog with a renal adenocarcinoma, but ALP and ALT were within reference limits (bilirubin concentration was not measured). The serum GGT activity, as measured by a different analyzer, was decreased after surgical removal of the neoplasm.[143] Increased serum GGT has been attributed to production by hepatocellular, renal, and other carcinomas in human patients,[144–146] and it is reportedly a negative prognostic indicator in dogs with hepatocellular carcinomas, but the pathogenesis of the increase has not been established.[147]

E. Bernese mountain dogs: Approximately 28 % of 32 clinically healthy Bernese mountain dogs had serum GGT activities greater than the URL of an established (details not provided) canine RI; the URL based on these 32 dogs was about 1.8 times that of the established canine RI. No pathologic cause of increased GGT activity was detected, suggesting a breed-specific physiologic difference necessitating a breed-specific RI.[100]

F. Idiopathic in racehorses:[148] Unexplained and reversible mild to moderate increases in serum GGT activity are reported in active racehorses, greater increases being associated with greater frequency of racing and greater overall training. Although other clear evidence of hepatic disease has not been evident from routine clinical biochemical testing, more specialized testing has provided evidence of hepatic disease, and roles for oxidative stress and cholestasis have been suggested.

VI. Species differences

A. Horses
1. Increased GGT activity has better diagnostic sensitivity than does ALP for detecting cholestasis or other biliary disorders in horses. In experimental cholestasis, both GGT

and ALP activities increased, but the GGT increase (~7×) was greater than the ALP increase (~2×).[141]

2. GGT may also increase with hepatocellular necrosis in horses.[141]
3. Neonatal foals have greater serum GGT activity than do their dams, but not because of GGT uptake in colostrum as occurs in ruminants and dogs.[132]

B. Cattle

1. Increased GGT activity is generally considered to have better diagnostic sensitivity than ALP for detecting cholestasis or other biliary disorders in cattle. Experimentally, GGT activity is increased in acute hepatic necrosis; the increase may reflect hepatocellular damage or secondary cholestasis.[8,141]
2. Disorders associated with increased GGT activity include bile duct obstruction, cholangitis, cholecystitis, copper toxicosis, hepatic mycotoxicosis, sporidesmin toxicity, *Brassica rapa* (turnip) toxicity, and fascioliasis.[149–153] GGT activity may be increased with hepatic lipidosis, but the magnitude is frequently mild.[154–156]

C. In camelids, GGT appears to be a useful marker of hepatobiliary disease. Llamas and alpacas with hepatic lipidosis tend to have increased GGT activity.[157,158]

D. In dogs, increased GGT activity tends to parallel increases in ALP activity caused by cholestatic disorders, but ALP probably has more diagnostic sensitivity and less diagnostic specificity for hepatobiliary disease.[159] GGT values can be increased in dogs with steroid hepatopathy.

E. In 54 cats with hepatic diseases, disorders associated with increased GGT activity included bile duct obstruction, cholangitis, cholangiohepatitis, lipidosis, neoplasia, hepatic necrosis, and cirrhosis. GGT activity in nine (17 %) of the cats was WRI.[119]

VII. GGT activity in urine

A. GGT has a molecular mass similar to albumin, so it does not pass freely through a healthy glomerular filtration barrier. However, damage to renal proximal tubular epithelial cells increases the urinary excretion of renal GGT without increasing the serum GGT activity.

B. Because the urinary GGT activity depends on the amount of H_2O excreted by the kidneys, the measured urine GGT activity is difficult to interpret. The $(GGT:Crt)_u$ ratio reduces the variations caused by variable degrees of renal tubular resorption of H_2O (see Chapter 8).

1. An increased $(GGT:Crt)_u$ ratio should indicate that the rate of GGT excretion is increased relative to the rate of Crt excretion. Such an increased $(GGT:Crt)_u$ ratio has occurred with renal diseases in dogs and ponies.[160]
2. Because renal Crt clearance depends almost entirely on GFR, but GGT excretion does not, an increased $(GGT:Crt)_u$ ratio might be caused by decreased GFR and not increased release of GGT by tubules.
3. The units for reported $(GGT:Crt)_u$ ratios have been mixed volume units: GGT measurement based on activity, Crt measurement based on mass. Different studies have used different units for Crt (mg/dL or g/L); therefore, it is important to check the units when comparing patient data to published studies or laboratory RIs.[161,162]

CREATINE KINASE (CK)

I. Physiologic processes, concepts, and facts

A. *CK* (EC 2.7.3.2) is a cytoplasmic enzyme that catalyzes a reversible phosphorylation reaction that converts creatine (not creatinine) and ATP to phosphocreatine and ADP. Creatine phosphokinase (CPK) is not an acceptable name for the enzyme.

B. Cytosolic CKs are dimers composed of combinations of muscle (M) and brain (B) subunits. There are three cytosolic CK isoenzymes that have variable cell distributions: CK-1 (CK-BB) predominates in brain and smooth muscle (e.g., intestinal), CK-2 (CK-MB) has significant presence in cardiac and smooth muscle, and CK-3 (CK-MM) predominates in striated and cardiac muscle.
C. Mitochondrial CKs (CK-Mt) are octameric isoenzymes present in many tissues.
D. In health, serum contains a mixture of CK isoenzymes, with CK-MM predominating in dogs and cats.[163] Differences in reported proportions in healthy animals may be due to different assay methods and their ability to detect macroenzymes. The diagnostic utility of measuring serum CK isoenzymes in domestic mammals is not established.
E. Young dogs have greater serum CK activity than adult dogs. The URL for CK activity in 16- to 24-d-old puppies is approximately 5.5 times the URL for adult dogs.[70] Similarly, the mean CK activity in puppies less than 1 mo old was found to be about 5× that of dogs > 1 yr old (236 U/L versus 46 U/L), while mean values in 1- to 6-mo-old puppies were 3.5–4 times as great (175 U/L), and 6- to 12-mo-old dogs had just over a 50 % increase (73 U/L).[164] The mean CK activity in small-breed dogs (< 10 kg) was about 1.7 times that of large-breed dogs (> 25 kg).[164] Physiologic explanations for the age and body size differences were not found. In healthy people, lean body mass does not appear to be a major factor in determining serum or plasma CK activity.[165]

II. Tissue sources of increased serum or plasma CK activity and CK half-life are listed in Table 12.2.

III. Stability: Canine CK activity in plasma was stable for 1 wk at –4 °C and for 1 mo at –20 °C.[164] It was also stable in serum and heparinized plasma for 3 d when stored at 4 °C on day 1 and at –20 °C on days 2 and 3.[25]

IV. Analytical concepts
A. The analytical ranges of routine commercial assays are too narrow for some domestic mammals, and thus samples frequently need to be diluted to obtain numeric results when CK activity is increased (numeric results may not be needed).
B. In samples from healthy dogs, the CK activity in serum is about 1.5–2.5 times that of plasma.[25,164] The difference may be caused by the release of CK from platelets during clotting, as canine platelets have been reported to contain CK activity.[166] This difference may not be clinically relevant when the increase in CK activity is moderate to marked. Incomplete removal of platelets from plasma could lead to mildly increased CK activity.

V. Increased serum or plasma CK activity (Table 12.8)
A. A variety of insults (pathologic and iatrogenic) may damage muscle fibers and cause them to release CK because of necrosis or reversible cell damage.
1. The magnitude of increase can be mild to extreme (< 2 × URL to > 1000 × URL) and is somewhat proportional to the degree of muscle damage, with mild increases being common and typically of little diagnostic value.
2. From a single insult (e.g., recumbency or other trauma), there can be a very rapid rise (hours) and a rapid decline (hours to days) because of the short CK half-life.
3. Myocardial damage may contribute to increased serum CK activity, but such contributions are typically speculative. Measurement of specific CK isoenzymes and troponin may provide more information about tissue source, but CK isoenzyme measurement has not been shown to be clinically useful in domestic mammals.
4. Mild increases may occur with myocyte damage from traumatic blood collection.

Table 12.8. Disorders or conditions that cause increased CK activity
Physiologic factors (puppies > adult dogs; small-breed dogs > large-breed dogs)
Muscle damage (mostly skeletal, occasionally cardiac, rarely smooth)*
Metabolic:* exertional rhabdomyolysis, hypothyroidism
Neoplastic: metastatic neoplasia
Nutritional: vitamin E or selenium deficiency
Inherited: some muscular dystrophies (dogs, cats), hyperkalemic myopathy, hypokalemic polymyopathies (e.g., in Burmese, Tonkinese, and Bombay cats), type 1 polysaccharide storage myopathy, malignant hyperthermia, myosin heavy chain myopathy in quarter horses and related breeds (nonexertional rhabdomyolysis), enzyme-deficiency myopathies (dogs, cats, cattle, horses)
Inflammatory: myositis caused by *Neospora*, *Toxoplasma*, bacteria, or other agents; bovine endometritis, endotoxemia;[167,168] immune-mediated myositis[169]
Toxic: monensin, castor bean, gossypol, box elder seeds,[170] rayless goldenrod (*Isocoma pluraflora*), goldenbush (*I. acradenia*), white snakeroot (*Ageratina altissima*), amphetamines (suspected in cats), sycamore (*Acer pseudoplatanus*) (atypical myopathy in horses)
Traumatic:* intramuscular injections, hit by car, gunshot, bite wounds, recumbency (especially horses and cattle), seizures, exertion
Vascular: saddle thrombus or other thromboembolism
Hemolysis, in vitro and potentially in vivo (some assays)

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Some conditions could fit into multiple DAMNITV categories.

B. Mild to marked increases in serum CK activity were reported in anorectic cats with nasoesophageal tubes and a wide variety of infectious, inflammatory, metabolic, or neoplastic disorders.[171] CK activities had decreased after 48 h of supportive alimentation, suggesting a relationship to caloric intake, but the decreases may have related to other treatments and factors as well. In a different study in which it was documented that increased serum CK activity is common in sick cats, CK activities did not appear to relate to anorexia.[172] The complete pathogenesis of increased CK activity in many sick cats is not known, but it presumably involves active muscle damage. Evidence to indicate that anorexia or muscle catabolism alone causes myocyte damage or increased serum CK activity is lacking.

C. Unexplained mild to at most moderate increases in serum CK activity have been reported in dogs and cats with no obvious insult to muscle but with a variety of metabolic disorders, particularly those involving metabolic acidosis.[173] Increased CK may relate to decreased CK clearance or myocyte damage induced or promoted by the underlying disorder, its sequelae, or its management.

D. Hypothyroidism in dogs is reported to cause increased CK activity, sometimes marked.[174] Whereas some hypothyroid dogs have increased serum CK activity, other hypothyroid dogs have decreased serum CK activity. Dogs with experimentally induced hypothyroidism developed mildly increased plasma CK activities (mean < 3 × URL) and subclinical structural and functional myocyte abnormalities.[49]

E. In vitro and potentially in vivo hemolysis may cause falsely increased CK activity, but the degree of interference varies with the method. RBCs do not contain CK but do contain glucose-6-phosphate, adenylate kinase, and ATP, which may interfere with the coupled reactions of CK assays.
F. Injury to organs and tissues containing smooth muscle may cause increased CK activity but to a lesser extent than with striated muscle damage. There is sufficient CK in the bovine uterus that endometritis can cause increased serum CK activity.[175] Four cows with moderate or severe endometritis had greater CK values (~7 or 15 times as high, respectively) than values found in five healthy cows. There was a concurrent, but relatively minimal, increase in AST activity.
G. Damage to brain tissue may cause increased CK activity in cerebrospinal fluid samples but does not usually cause increased CK activity in serum or plasma. In most cases, increased CK activity in domestic mammals with neurologic disease appears to result from the release of muscle CK secondary to convulsions or recumbency. A dog with an intracranial mass and mildly increased serum total CK activity (112 % of the highest value of 20 healthy dogs) had serum CK-BB that was 3.75 times the maximum value for 20 healthy dogs, while CK-MM was just 2 % higher and other subtypes were not increased; this suggests that CNS disease may have contributed CK-BB to the mild increase in total CK activity.[163]
H. Increased CK activity is not expected with uncomplicated disuse atrophy of muscle.

α-AMYLASE

I. Physiologic processes, concepts, and facts
A. *α-Amylase* (EC 3.2.1.1) is a cytoplasmic enzyme that requires Ca^{2+} and Cl^- as cofactors and catalyzes the hydrolysis of complex starches.
B. Via electrophoresis, four α-amylase isoenzymes (I–IV) are classified in dogs; isoenzyme IV includes macroamylases, which are protein complexes that contain α-amylase bound to immunoglobulins or other proteins.
C. People and pigs have salivary α-amylase, whereas dogs,[176] cats, and cattle do not. A salivary α-amylase gene has been identified in horses,[177] but equine salivary gland tissue appears to have relatively low amylase activity,[178] and reports of equine salivary α-amylase secretion were not found. Data were not found for llamas and alpacas.
D. α-Amylase activity in serum of healthy dogs includes pancreatic α-amylase, intestinal α-amylase, and macroamylase activities. The liver may also contribute.
1. Compared to presurgical measurements, serum α-amylase activity in two of five dogs did not decrease by 7 d after pancreatectomy. In three of five dogs, serum α-amylase activity decreased by about 50 %. Therefore, in healthy dogs, serum α-amylase activity may be from pancreatic and nonpancreatic tissues.[179]
2. Up to 70 % of the plasma α-amylase in healthy dogs was of intestinal origin;[180] however, intestine is not known to be a source of increased serum α-amylase activity. In another group of healthy dogs, about 14 % of total α-amylase was bound to immunoglobulins (macroamylase).[181]
E. Some investigators have found α-amylase activity and mRNA for α-amylase in canine liver,[180,182–185] so the liver might contribute to baseline serum α-amylase activity without storing appreciable amounts of the enzyme. Further observations support this idea: (i) serum α-amylase activity is sometimes less than the lower reference limit in dogs with portosystemic shunts (authors' observation), and (ii) serum α-amylase activity decreased in rats after liver

damage or inhibition of hepatic α-amylase synthesis. However, there is not good evidence that hepatic disease increases serum α-amylase activity.

F. The kidneys are a route of plasma α-amylase excretion or inactivation (see α-Amylase, Section V.B), but there is experimental evidence that other routes, including hepatic macrophages, may also be involved.[186,187] Although the former is of clinical diagnostic relevance, clinical relevance of hepatic clearance has not been shown.

II. Tissue sources of increased serum or plasma α-amylase activity and half-life are listed in Table 12.2.

III. Stability: α-Amylase activity of healthy dogs was stable for 3 d in serum and heparinized plasma samples stored at 4 °C on day 1 and at −20 °C on days 2 and 3.[25]

IV. Analytical concepts

A. α-Amylase used to be a standard component of small animal chemistry profiles, but it became inconsistently offered once testing for PLI became more available (see Chapter 15).

B. Three major types of assays have been used to measure α-amylase activity.

1. In saccharogenic assays (*saccharo-* means "sugar" and *-geny* means "produce"), α-amylase catalyzes the degradation of starch to produce glucose and maltose, which are coupled with other measurable reactions to reflect α-amylase activity. Canine serum contains glucoamylase and maltase (synonym: α-glucosidase) that may falsely increase α-amylase activity by reacting with the maltose produced by the α-amylase reaction.[188]
2. In amyloclastic assays (*amylo-* means "starch" and *-clast* means "breaking"), a dye binds to the available starch. If there is less bound dye at the end of the assay, then there was more α-amylase activity. Glucoamylase is not an interferent, but these assays are not easily automated.
3. In chromogenic assays, which are most common, α-amylase catalyzes the cleavage of a dye bound to a synthetic substrate, and the change in dye absorption is measured. Some chromogenic assays detect nonpancreatic glucoamylase and therefore are not acceptable for measuring canine α-amylase.[189]

C. Specific α-amylase isoenzymes can be measured, but their clinical value is limited.

V. Increased serum or plasma α-amylase activity (Table 12.9)

A. Pancreatic acinar cell damage

1. Acute pancreatitis is the most common cause of damage to pancreatic acinar cells, and it may result in the release of α-amylase, especially in dogs. The damage may lead to acinar cell necrosis or pancreatic abscess formation. The α-amylase may gain access to blood via veins draining the pancreas and via lymphatic vessels.[190] In experimental canine pancreatitis, serum α-amylase activity peaks within 12–48 h and persists for 8–14 d.[191]
2. Species variations
   a. Serum α-amylase activities in dogs with acute pancreatitis range from WRI to extremely increased (> 10 × URL). In one study, increases were present in most dogs with histologically confirmed chronic pancreatitis, but they were usually mild (< 3 × URL).[192]
   b. Serum α-amylase activity is of little clinical value for diagnosing pancreatitis in cats. In cats with spontaneous pancreatitis, serum α-amylase activities range from WRI to mildly increased (< 3 × URL).[193,194] In experimental pancreatitis in six cats, serum α-amylase activity did not increase.[195]

Table 12.9. Disorders or conditions that cause increased α-amylase and lipase activities
Pancreatic acinar cell damage:* inflammation, primary or secondary pancreatic neoplasms (α-amylase and lipase); trauma (lipase)
Decreased renal inactivation or clearance
Prerenal disorders: dehydration, shock (α-amylase and lipase)
Renal disorders:* acute or chronic renal diseases (α-amylase and lipase)
Postrenal disorders: urinary tract obstruction (α-amylase and lipase)
Macroamylasemia (α-amylase)
Other or unknown mechanism (see text for lipase)
Pancreatic or hepatic neoplasms (lipase, possibly α-amylase)
Exogenous or endogenous glucocorticoids (lipase)
Breed variation: Bernese mountain dogs (α-amylase)

* A relatively common disorder, condition, or mechanism

c. Although α-amylase is not often assessed in horses because of the rarity of equine pancreatic disease, and consequently appropriate laboratory reference values may not be available, hyperamylasemia has been reported in foals with acute pancreatitis.[196]
d. Measurement of serum α-amylase activity has not been useful for diagnosing pancreatitis or other diseases in cattle.
e. Serum α-amylase activity was reportedly increased in three of seven llamas or alpacas with pancreatic necrosis or pancreatitis, and five of seven had greater α-amylase activity in peritoneal fluid than in serum (RI source and values were not provided).[197] In contrast to the latter, serum α-amylase activity was about 80 % greater than α-amylase activity in concurrently collected peritoneal fluid from 23 healthy llamas and alpacas.[198]

3. Pancreatic neoplasms can also lead to increased serum α-amylase activity, whether through acinar cell damage or increased synthesis and release.[199]

B. Decreased renal inactivation or excretion

1. In dogs, clinical and experimental data indicate that the half-life of plasma α-amylase increases when renal blood flow or functional renal tissue decreases.[4,200] Experimentally, the increased half-life may lead to hyperamylasemia (mean increase, 2.5–4.0 × baseline values). Clinically, the increased α-amylase activity is expected to be < 3 × URL in conditions that cause decreased GFR without pancreatic acinar cell damage.
2. Although decreased renal clearance or inactivation contributes to hyperamylasemia during renal failure or states of diminished renal blood flow, exactly how α-amylase is handled by the kidneys has not been established.
   a. Very little to no α-amylase activity is found in the urine of healthy dogs, perhaps because α-amylase ($M_r \approx 55{,}000$) does not pass through the glomerular filtration barrier but is removed from blood or inactivated as it passes through the renal vasculature, or because it is inactivated or resorbed by tubular cells after it passes through the barrier.
   b. There is a correlation between amylasuria and proteinuria, especially glomerular proteinuria. Thus, it appears in health that the glomerular filtration barrier limits the amount of α-amylase that reaches the tubular lumen, or perhaps a large amount of luminal protein resulting from glomerular disease interferes with α-amylase inactivation by the tubular epithelial cells.[181,201,202]

c. Experimental damage to proximal renal tubular cells increased urinary loss of α-amylase, but relatively not as much as urinary loss of lipase or lysozyme. Proximal tubular epithelial cells have some capability of inactivating or resorbing α-amylase if α-amylase does pass through the glomerular filtration barrier.[203]

d. Formation of macroamylase molecules in blood also influences serum α-amylase activity. *Macroamylase*, a complex between α-amylase and an immunoglobulin or other protein, is too large to pass through a healthy glomerular filtration barrier and thus may have a longer half-life than noncomplexed α-amylase. Macroamylasemia contributes to the hyperamylasemia in some proteinuric and azotemic dogs; however, hyperamylasemia was still present in the serum of some of these dogs after the macroamylase molecules were removed.[181]

3. The importance of renal inactivation or excretion of α-amylase by feline kidneys has not been established. Of 32 cats with renal failure, 10 had hyperamylasemia (slight to 3 × URL).[204]

C. Bernese mountain dogs: Approximately 41 % of 32 clinically healthy Bernese mountain dogs had serum α-amylase activities greater than an established (details not provided) canine RI; the URL based on these 32 dogs was about 1.6 times that of the established canine RI. No pathologic cause of increased amylase activity was detected, suggesting a breed-specific physiologic difference necessitating a breed-specific RI.[100]

D. Intestinal α-amylase has not been shown to increase total serum α-amylase activity; cytoplasmic and membrane-expressed α-amylase is expected to enter the intestinal lumen with cell turnover.

LIPASE

I. Physiologic processes, concepts, and facts

A. Lipase enzymes catalyze hydrolysis of acylglycerols (fatty acid esters of glycerol), most relevantly triacylglycerols, which are also known as triglycerides (TGs).

B. Several lipases are in the body, and each has specific roles in lipid metabolism. More information about lipid metabolism is in Chapter 16.

1. Triacylglycerol lipases, each produced from a different gene (EC 3.1.1.3)

a. *Pancreatic lipase* (pancreatic triacylglycerol lipase) is produced by pancreatic acinar cells[205] and catalyzes the hydrolysis of dietary TG in the intestine, generating two fatty acids per triacylglycerol molecule. Pancreatic lipase requires Ca^{2+}, colipase, and bile salts as cofactors for optimal activity. The pancreatic lipase molecule ($M_r \cong 51{,}000–52{,}000$, dogs and cats) and colipase are small enough to pass through the glomerular filtration barrier to be inactivated or excreted.

b. *Gastric lipase* (gastric triacylglycerol lipase) is produced by gastric mucosal cells[205] and can remove one fatty acid from each ingested TG. It is an acidic lipase and, in contrast to pancreatic lipase, it does not require bile acids and colipase for optimal activity.

c. *Hepatic lipase* (hepatic triacylglycerol lipase) is produced by hepatocytes, is present on hepatocytes and endothelial cells of hepatic sinusoids, and hydrolyzes TG in some lipoprotein molecules.

2. Many extrahepatic cells, including adipocytes and myocytes, produce *lipoprotein lipase* (EC 3.1.1.34), which, under the influence of insulin, migrates to the luminal surface of endothelial cells, where it catalyzes the hydrolysis of TG in plasma lipoproteins (e.g., chylomicrons and very low-density lipoproteins).

3. *Hormone-sensitive lipase* (EC 3.1.1.79) is a neutral lipase in adipocytes that catalyzes the hydrolysis of stored TG and the liberation of fatty acids; it has a broad substrate specificity and is stimulated by epinephrine and glucagon.
4. *Lysosomal acid lipase* (sterol esterase, EC 3.1.1.13) is an intracellular lipase that catalyzes the hydrolysis of cholesterol esters.

C. Compared to presurgical measurements, serum lipase activity in four of five dogs decreased 50–75 % by day 7 after pancreatectomy. However, lipase activity increased by 40 % for one dog in the same study. Data indicated that the pancreas was a contributor to serum lipase activity, but there were other tissue sources in some dogs.[179] The nonpancreatic lipase in serum might be gastric lipase or hepatic lipase.

D. As lipase activities in serum or plasma may involve multiple lipase enzymes, the generic term "lipase" will be used throughout.

II. Known tissue sources of increased serum or plasma lipase activity and lipase half-life are listed in Table 12.2.

III. Stability: Lipase activity of healthy dogs was stable for 3 d in serum and heparinized plasma samples stored at 4 °C on day 1 and at –20 °C on days 2 and 3.[25] Good precision was maintained for serum samples stored for 20 d at –20 °C.[206] Lipase assay package inserts from some companies indicate stability in frozen samples (≤ –20 °C) for a year or longer, but published data were not found.

IV. Analytical concepts

A. Lipase is assessed either by enzymatic lipase assays that measure catalytic activity (unit = U/L) or by immunoassays that measure mass concentration of the lipase protein(s) targeted by assay antibodies (unit = µg/L). These assays and their use to assess dogs and cats for evidence of pancreatitis have been reviewed.[207–209] However, it should be noted that the many publications regarding clinical performance of these assays are difficult to assess and compare for many reasons, including (i) lack of complete analytical validation of some assays, (ii) inadequate precision of some assays, (iii) variation in instrumentation and assays, even for assays based on the same principle (see first section of this chapter, Basic Principles in Clinical Enzymology, IV), (iv) use of samples stored for longer periods than documented to maintain stability, (v) varied diagnostic criteria for pancreatitis, (vi) varied populations of animals considered healthy, sick with pancreatitis, or sick with diseases that cause similar presentations, (vii) unclear quality of RIs, and (viii) varied decision limits (cutoffs) to interpret values as being increased or supportive of pancreatitis.

B. Enzymatic lipase assays

1. Clinical enzymatic lipase assay methods have yielded disparate results partly because of variations in analytical specificity for pancreatic lipase. Earlier assays using olive oil or 1,2-diglyceride (DiG) as substrates were less specific for pancreatic lipase, whereas more recent assays using DGGR or triolein as substrates are generally more specific for pancreatic lipase.[206,210] Use of exogenous Ca^{2+}, bile salts, and colipase promotes measurement of total pancreatic lipase activity, as does the anionic detergent sodium dodecyl benzene sulphonate for a triolein-based assay. However, there is evidence that DGGR and triolein assays may detect other lipases.
   a. Very mild and transient increases in lipase activity (DGGR assay) compared to baseline occurred after heparin administration to dogs and cats.[211] This activity

was likely from hepatic lipase, lipoprotein lipase, or both, because exogenous heparin releases these enzymes from cell membranes into circulation.

b. Dogs with exocrine pancreatic insufficiency had more serum lipase activity by DGGR and triolein methods than investigators expected if only pancreatic lipase was assessed, but all results were WRI and most were in the lower half of the RIs.[212]

c. However, the degree to which extrapancreatic lipases actually contribute to increased lipase activity when clinical samples are assessed by DGGR and triolein assays is unknown, so it is not clear that extrapancreatic lipases cause diagnostic problems. In fact, when appropriate decision limits are used, these assays have yielded results that classified dogs and cats similarly to classifications based on PLI results (i.e., supportive of pancreatitis, no support for pancreatitis, or equivocal).[213–218]

2. DGGR assays have been used in horses.[219]

3. Interferences: Published data are very limited, but hemolysis and lipemia reportedly had no significant effect on results of one DGGR assay for dogs.[206] With human samples, hemolysis and bilirubin did not interfere with a DGGR assay, but interference was reported when TG concentrations were > 500 mg/dL.[210] With a triolein assay (v-LIP-P), lipase activity was falsely decreased in canine samples with added hemoglobin or ditaurobilirubin to mimic hemolysis and icterus; effects of lipemia were less clear.[212]

C. PLI assays: Species-specific assays for dogs (cPLI or cPL) and cats (fPLI or fPL) measure the serum or plasma concentration of proteins recognized by antibodies targeting pancreatic lipase protein. These include canine and feline in-house semiquantitative ELISAs (IDEXX SNAP PL, Abaxis VETSCAN cPL Rapid Test) and quantitative in-house (Bionote Vcheck cPL and fPL, fluorescence immunoassays) or reference laboratory ELISAs using monoclonal antibodies (IDEXX Spec PL) or polyclonal antibodies (Laboklin GmbH & CO KG). More information about pancreatic lipase immunoassays is in Chapter 15.

V. Increased serum or plasma lipase activity (Table 12.9)

A. Pancreatic acinar cell damage

1. Acute pancreatitis is the most common cause of damage to pancreatic acinar cells and will result in the release of lipase, especially in dogs. The damage may or may not cause acinar cell necrosis. Acinar cell necrosis and pancreatitis can occur because of primary or rarely secondary pancreatic neoplasms (e.g., pancreatic acinar cell carcinomas, lymphoma). The lipase may gain access to blood via lymphatics or possibly directly through small blood vessels with altered permeability because of local inflammation.

2. Serum lipase activity was mildly increased (< 4 × URL) in two of three cats with pancreatic rupture from high-rise syndrome (falling from the second floor of a building or higher), and lipase activity in peritoneal effusions was > 20 times the serum lipase activity in all three cats.[220]

3. Species variations

a. Serum lipase activity in dogs with acute pancreatitis ranges from WRI to extremely increased (> 10 × URL). In one study, mild to marked increases were present in most dogs with histologically confirmed chronic pancreatitis.[192] The reported diagnostic sensitivity and specificity of serum lipase activity for canine pancreatitis have varied widely from moderate to high. Reasons for these wide variations include use of different assays, different diagnostic criteria, and different populations of dogs.

b. In spontaneous feline pancreatitis, serum lipase activity ranges from WRI to moderately increased (< 5 × URL).[193,194] In experimental pancreatitis in six cats,

serum lipase activity was increased up to 6 × baseline values for the first day and then reduced to ~2 × baseline values for 1 wk.[195]

c. Historically, lipase was not often assessed in horses because of the rarity of recognized equine diseases causing hyperlipasemia, but moderate (~5 × URL) to marked (> 10 × URL) hyperlipasemia (based on limited reference values) has been reported in foals with acute pancreatitis.[196] More recently, increased plasma lipase activity was reported in many horses with various gastrointestinal disorders, particularly large bowel displacement or torsion, but pancreatic lesions were not detected in the few affected horses that underwent necropsy and the cause is not established.[178,219] Additionally, the quality of RIs for these studies is unclear.

d. Measurement of serum lipase activity has not been useful for diagnosing pancreatitis or other diseases in cattle.

e. Serum lipase activity was an insensitive marker of pancreatic necrosis in llamas and alpacas, being increased in only one of seven reported cases (RI source and values were not provided).[197] Lipase activity in all seven of these camelids was greater in peritoneal fluid than in serum, whereas serum lipase activity was about 40 % greater than peritoneal fluid lipase activity in a group of 23 healthy alpacas and llamas.[198] Finding a greater lipase activity in peritoneal fluid compared to serum may be supportive of pancreatic acinar cell damage.

B. Decreased renal inactivation or excretion

1. The exact mechanism by which lipase is handled by the kidneys is not clear but appears to involve glomerular filtration, tubular resorption, and enzyme inactivation. With impairment of these functions, hyperlipasemia may occur. However, it is inconsistently present in patients with renal disease and may involve other mechanisms, including concurrent pancreatic acinar cell damage. Clinically, lipase activity is expected to be < 4 × URL in renal disease without pancreatic acinar cell damage.
2. With early less-specific olive-oil-based lipase assays, serum lipase activity tended to parallel serum α-amylase activity in dogs with decreased renal blood flow or decreased functional renal tissue from experimentally induced or naturally occurring disease.[4,200] Experimentally, some dogs developed hyperlipasemia (mean increase, 2.5–4.0 × baseline values, often not > URL) that did not correlate with azotemia. However, greater lipase activity (6–8 × URL) was reported with some renal cases that did not have evidence of pancreatic disease,[221] and a lipase activity of 10 × URL was reported in a case of spontaneous primary renal failure.[200]
3. In a small study using a DiG-substrate assay, reversible gentamicin-induced acute renal failure yielded azotemia without parallel increases in serum lipase activity; a small percentage of samples had increased lipase activity (and PLI concentration).[222] The cause of these increases is unknown.
4. Renal disease was a common diagnosis in a retrospective study of critically ill dogs with hyperlipasemia (some > 3 × URL) using a DGGR-substrate assay (there were similar increases in PLI), but the correlation between DGGR-lipase activity and [Crt] was poor.[214] It is unknown if these dogs had concurrent pancreatitis, altered renal metabolism of lipase, or other causes of hyperlipasemia (e.g., glucocorticoid treatment).

C. Proliferation of cells containing lipase activity: Pancreatic neoplasms and extrapancreatic neoplasms (e.g., cholangiocarcinoma) can cause mildly to markedly increased serum lipase activity.[223] In five of six cases, histochemical and immunohistochemical findings indicated that the neoplasms were potential sources of the serum lipase, and the same dogs did not have

significant increases in serum α-amylase activity.[224] Some of the serum activity may stem from enzyme release from damaged cells, and concurrent pancreatitis may contribute.[176]

D. Glucocorticoids: Exogenous or endogenous glucocorticoids have been associated with hyperlipasemia in dogs without other clinical evidence of pancreatitis. Increases might reflect subclinical pancreatitis or changes in lipase production, secretion, or clearance.
   1. Dexamethasone: Using an olive-oil-substrate lipase assay, mild hyperlipasemia (< 2 × URL) was found in 24 healthy dogs by day 8 of treatment with dexamethasone either at 2 mg/kg or 0.2 mg/kg. In dogs with neurologic disease, hyperlipasemia of increasing severity (mean value of 4.6 × URL) occurred by day 10 of treatment (initial dose at 2 mg/kg and then reduced doses). The number of dogs sampled varied from two to eight on different days. Neither microscopic evidence of pancreatic damage nor concurrent hyperamylasemia was found.[225]
   2. Prednisolone or prednisone
      a. Mildly increased serum lipase activity (DGGR assay) was measured in a small minority of 17 dogs prescribed prednisolone for nonpancreatic disease (0.5–2.0 mg/kg/d orally for 1 wk), but activities were not increased after a second week at about half the dose, and no values exceeded the authors' medical decision limit (2 × URL) for pancreatitis.[226] Healthy dogs treated similarly did not have increased serum lipase activity. It is not known if these mild increases reflected occult pancreatitis or other processes related to prednisolone or underlying disease.
      b. One of six healthy beagles treated subcutaneously for 2 or 3 wk with prednisolone (4 mg/kg/d) had increased serum lipase activity on day 14 (v-LIP-P triolein assay) without clinical signs of pancreatitis.[227] In a different study, 5 of 10 dogs with various immune-mediated disorders for which oral prednisolone was administered (initial dose of 2.0–2.2 mg/kg/d) developed increased serum PLI concentrations (Spec cPL) without clinical pancreatitis.[228] PLI peaked at weeks 1, 2, 6, or 7 of therapy, which was after dose reductions for two dogs. In contrast, in a third study, PLI increases were not detected with oral prednisone (2.2 mg/kg/d for 4 wk) in six female dogs with X-linked hereditary nephritis.[229]
   3. Hyperadrenocorticism: In a study of 22 dogs with hyperadrenocorticism, DGGR-lipase activity was increased in 64 % of the dogs and DiG-lipase was increased in 23 % of them with no other clinical evidence of acute pancreatitis.[230] Most increases were mild. However, 18% of healthy dogs had a DGGR-lipase activity > URL, suggesting that the reference limit might not have been appropriate. In another study of 20 dogs with hyperadrenocorticism and no clinical evidence of acute pancreatitis, 12 had increased PLI concentrations, 7 of which were high enough to support pancreatitis (> 400 µg/L); only 1 control dog had increased PLI.[231] It is not known if hyperlipasemia in dogs with hyperadrenocorticism reflects subclinical pancreatitis or other changes in lipase production, secretion, or clearance.

E. Other disorders or states: Hyperlipasemia based on lipase activities or concentrations has been reported in dogs with many other conditions and no other clinical evidence of acute pancreatitis. Potential causes include concurrent primary or secondary subclinical pancreatic injury, increased extrapancreatic lipases, inappropriate RIs, and various analytical errors such as assay imprecision. In many cases, pancreatic hypoperfusion or hypoxia might be involved.
   1. DGGR-lipase activity was > 3 × URL in 16 % of 1360 critically ill dogs with many different disorders, and 146 of these 216 dogs (67 %) did not have a clinical diagnosis of acute pancreatitis.[214] PLI results corroborated lipase activity results. Diagnoses with the

greatest percentage of lipase activities > 3 × URL were renal disease ± hemodialysis, endocrinopathies (mostly diabetes mellitus and hypoadrenocorticism), immune-mediated disorders, and upper airway obstruction. Prior glucocorticoid use might have contributed (e.g., in dogs with immune-mediated disease and hypoadrenocorticism).

2. Other conditions for which increased lipase activity or concentration have been reported in some dogs include congestive heart failure, mitral valve disease, gastroenteritides (including mushroom toxicosis), gastric dilation and volvulus, intervertebral disk disease, monocytic ehrlichiosis, hemodialysis for acute renal disease, and portal hypertension with hepatopathy.
3. Human conditions causing hyperlipasemia without pancreatitis are similar and have been reviewed.[232] These include idiopathic chronic nonpathologic pancreatic hyperenzymemia characterized by chronic increases in amylase and lipase without clinical disease.[233]

THYMIDINE KINASE (TK)

I. Physiologic processes, concepts, and facts
   A. *TK* (EC 2.7.1.21) catalyzes a reversible reaction that converts thymidine and ATP to thymidine 5'-phosphate and ADP prior to formation of thymidine triphosphate, which is incorporated into DNA. The two isoenzymes of TK, TK1 and TK2, are deoxyribonucleoside kinases involved in recycling deoxyribonucleosides for DNA synthesis.[234]
   B. TK1 is a cytoplasmic enzyme widely expressed in neoplastic and nonneoplastic cells, but only while they are proliferating. In nonneoplastic cells, intracellular activity surges in the S phase of the cell cycle and rapidly decreases as TK1 is degraded intracellularly. In neoplastic cells, TK1 expression can remain high in other phases of the cell cycle.
   C. TK2 is a mitochondrial enzyme present in cells in proportion to mitochondrial content; it is not regulated by the cell cycle.[234,235]

II. Stability: No significant difference in serum TK activity was found in canine samples stored for 24 h at room temperature, for 24 h at 37 °C, or for 3 mo at −20 °C.[236] However, substantial false increases appear to occur if serum is not promptly separated from clotted canine blood.[237]

III. Analytical concepts
   A. TK1 activity in serum or heparinized plasma of domestic mammals has been measured by radioenzymatic, enzyme-linked immunosorbent, and enzyme-linked chemiluminescent assays. Selectivity for TK1 is based on different substrate specificities for TK1 and TK2. Immunoassays that measure TK1 mass concentration instead of activity concentration have also been used, and they can detect both active and inactive forms.[238–240] Results of an activity assay and a mass concentration assay paralleled each other in a study of dogs with hemic neoplasms, mostly lymphoma.[241]
      1. Disadvantages of activity assays include (i) cross-reactivity of some assays with TK2,[242] (ii) inhibition by TK1 inhibitors in plasma or serum, and (iii) inability to detect inactive forms of TK1.[243]
      2. Mass concentration immunoassays overcome these problems and may be less susceptible to sample processing errors, but they require species-specific antibodies of appropriate molecular specificity. Also, their results may be affected by the various sizes of TK1 complexes that form in circulation, though this problem is reduced by use of a reagent to break down the complexes before analysis.[243]

Table 12.10. Disorders or conditions that cause increased serum or plasma thymidine kinase activity
Neoplasms
Lymphoma*
Leukemia
Other solid tissue neoplasms
Infectious / inflammatory diseases

* A relatively common disorder, condition, or mechanism

B. Catalytic activity units have varied (e.g., U/L versus pmol/min/mL), as have mass concentration units (pM versus ng/mL), so it is important to check the units and compare to the appropriate URL to determine if TK activity is increased. Decreased TK activity cannot be assessed with these assays and is not of diagnostic interest.[238]

IV. Increased serum or plasma TK activity (Table 12.10)
- A. The cellular mechanism(s) responsible for increased TK activity are unknown. Theories include release from intact dividing cells and release associated with death of proliferating cells.[244,245]
- B. Neoplasms
  1. Lymphoma: Serum TK activity has been investigated mainly as a biomarker for lymphoma, and it may be useful for monitoring clinical remission and recurrence of lymphoma in those patients with increased pretreatment serum TK activity.[236,246]
     a. Increased TK activity has been reported in dogs, cats, horses, and cattle with lymphoma.[236,241, 245–250] The magnitude of increase tends to be greater than that of increases associated with most other conditions. Inconsistent findings for equine lymphoma might relate to the different assays used or to differences in sample processing (details were not provided).[247,249,251]
     b. Many lymphoma patients do not have increased serum TK activity, and those that do may have mild to marked increases. Results in cows using decision limits (not RIs) suggest that TK activity may have particular diagnostic value in this species.[248]
     c. Serum TK activity decreases with remission of lymphoma in dogs.[236,241,246,252–254]
  2. Leukemia: Moderately to markedly increased serum TK activity was present in all 35 dogs diagnosed with leukemia (types not detailed but included acute and chronic myeloid leukemias); TK activities were lower in the subgroup of dogs that were in remission.[252]
  3. Other solid tissue neoplasms: As in people, increased TK activity has been reported in cats and dogs with a variety of carcinomas and sarcomas,[237,253,255,256] as well as melanomas and mast cell neoplasms.[237] The magnitude of increase tends to be lower than for lymphoma and leukemias.[253,256]
- C. Nonneoplastic disease: Serum TK activity may be increased in association with inflammatory diseases such as pancreatitis in cats and pyometra in dogs.[239,256,257]

OTHER SERUM ENZYMES

Many enzymes present in serum or plasma have been assessed in an attempt to find better indicators of pathologic states involving hepatocytes or other cells (Table 12.11). For a variety of reasons, most have failed to be as clinically valuable as those described earlier in this chapter.

Table 12.11. Other serum enzymes and enzymatic proteins

Serum enzyme	EC number	Change with disorders	Diagnostic value
5′-Nucleotidase	3.1.3.5	↑	Hepatobiliary disease
α-Glucosidase (maltase)	3.2.1.20	↑	Intestinal mucosa damage
Arginase	3.5.3.1	↑	Hepatocyte damage
Cholinesterase[a]	3.1.1.8	↓	Organophosphate toxicosis, infections, chronic hepatic disease
Fructose-bisphosphate aldolase	4.1.2.13	↑	Myocyte damage
Isocitrate dehydrogenase	1.1.1.42	↑	Hepatocyte damage
Leucyl aminopeptidase[b]	3.4.11.1	↑	Hepatobiliary disease
Lysozyme (muramidase)	3.2.1.17	↑	Neoplasia (histiocytic)
Malate dehydrogenase	1.1.1.37	↑	Hepatocyte damage
Ornithine carbamoyltransferase	2.1.3.3	↑	Hepatocyte damage
Trypsin-like immunoreactivity (TLI) (see Chapter 15)	—	↑	Pancreatitis or decreased GFR
		↓	Exocrine pancreatic insufficiency in dogs and cats

[a] Plasma pseudocholinesterase or butyrylcholinesterase, not acetylcholinesterase of the myoneural junction
[b] Also called leucine aminopeptidase

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 13

HEPATOBILIARY FUNCTION

Michael A. Scott, Michael M. Fry, and Steven L. Stockham

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Table 13.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
ALP	Alkaline phosphatase
AST	Aspartate transaminase
Bδ	δ-Bilirubin (Bc covalently bound to albumin)
BA	Bile acid
Bc	Conjugated bilirubin
Bd	Direct bilirubin
Bi	Indirect bilirubin
Bt	Total bilirubin
Bu	Unconjugated bilirubin
Bu/Alb	Unconjugated bilirubin bound to albumin (noncovalent)
GGT	γ-Glutamyltransferase
GMD	Glutamate dehydrogenase
HCO_3^-	Bicarbonate
Hgb	Hemoglobin
ID/SDH	Iditol dehydrogenase, also known as sorbitol dehydrogenase
LD	Lactate dehydrogenase
NH_3	Ammonia
NH_4^+	Ammonium
RBC	Red blood cell (erythrocyte)
RI	Reference interval
SI	Système international d'unités (International System of Units)
URL	Upper reference limit
USG_{ref}	Refractometric urine specific gravity

HEPATOBILIARY DISEASE AND HEPATIC INSUFFICIENCY

The *hepatobiliary system* includes the liver (hepato-) and the *biliary tree*, which consists of components within the liver (bile canaliculi, ductules, and ducts) and outside it (common bile duct and, in most species, gall bladder). The term *hepatic* refers to all parts of the liver, not just hepatocytes, and it does not include the gall bladder or common bile duct. When severe enough, disorders of the hepatobiliary system alter body fluids and cause changes in routine laboratory test results. Although each such change by itself is not specific for hepatobiliary disease, a constellation of abnormal findings may strongly suggest hepatobiliary disease, whether primary or secondary, and direct further, more-specialized testing of liver function along with tests for underlying disorders. This chapter focuses on bilirubin and the ancillary laboratory tests that are used to further support liver malfunction. It is first important to recognize the different types of hepatobiliary disorders and the terminology used to discuss them.

I. The term *hepatobiliary disease* refers herein to any disorder affecting any part of the liver or extrahepatic biliary tree, though the term may also be used to describe disorders that affect the biliary tree and liver together. Hepatobiliary disease may be primary, but it is often secondary and

related to underlying or systemic disorders. Some pathologic processes primarily affect hepatocytes, others primarily affect the biliary system, and some affect both. Recognizing which pattern is present helps to refine a differential diagnosis. However, primary *hepatocellular disease* – disease of hepatocytes – often leads to secondary biliary disease, and primary biliary disease often leads to secondary hepatocellular disease, so a distinction is not always apparent. If hepatocellular disease becomes widespread and severe enough, it may cause liver insufficiency or failure.

II. Laboratory abnormalities can provide evidence of the following three general pathologic hepatobiliary states, which may occur independently or together:
   A. *Hepatic insufficiency*, often considered synonymous with *liver failure*, is a pathophysiologic state in which the number of functioning hepatocytes is markedly reduced. Like many other organs, the liver has a large functional reserve; hepatic insufficiency is typically not detected until there is at least a 70 % loss in hepatic functional mass (hepatocytes). This may be associated with an abnormally small liver (microhepatica) resulting from too few hepatocytes, or with a normal-sized to enlarged liver with many dysfunctional hepatocytes.
      1. The many disorders that can decrease functional hepatic mass (Table 13.2) fall into two groups:
         a. Disorders that destroy hepatocytes: These may progress slowly, recur episodically, or involve rapid, extensive necrosis.
         b. Vascular disease including portosystemic shunts (congenital or acquired) and hepatic microvascular dysplasia: These cause hypoplasia or atrophy of the liver because of decreased nutrients reaching hepatocytes from portal blood.
      2. Alterations in blood and urine constituents caused by insufficient functional hepatocytes or decreased portal blood to the liver lead to laboratory test abnormalities that together may support hepatic insufficiency. However, they do not indicate the underlying disorder or whether the disease process is reversible or irreversible.
   B. *Hepatocellular disease*, a general descriptive term for hepatocyte damage, may occur with a wide variety of disorders (i.e., degenerative, autoimmune, anomalous, metabolic,

Table 13.2. Disorders and conditions that cause decreased functional hepatic mass (using the *DAMNIT* acronym)

*D*egenerative: hypoxia caused by anemia or congestion
*A*nomalous: portosystemic shunt,* hepatic microvascular dysplasia
*M*etabolic: lipidosis, diabetes mellitus, hyperadrenocorticism
*N*eoplastic: lymphoma, metastatic neoplasms
*I*nflammatory
Infectious: leptospirosis, histoplasmosis, feline infectious peritonitis
Noninfectious: chronic active hepatitis*
*I*nherited: copper or lysosomal storage diseases
*T*oxic:* steroid hepatopathy, some anesthetic agents, tetracycline, carprofen, copper toxicosis, hemochromatosis, pyrrolizidine alkaloid–containing plants
*T*raumatic: hit by car

* A relatively common disorder, condition, or mechanism of clinical hepatic dysfunction
Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Some conditions could fit into multiple DAMNIT categories.

nutritional, neoplastic, inflammatory, infectious, inherited, toxic, or traumatic). It is typically recognized by increased serum or plasma activities of enzymes released from hepatocytes because of hepatocyte injury (see Chapter 12).

1. Assays for these enzyme activities are not considered hepatic function tests, as the enzymes are not solely of hepatic origin, and they do not directly indicate loss of any hepatic synthetic or metabolic function.
2. Many animals with hepatocellular disease do not have enough hepatocyte damage to cause hepatic insufficiency. In contrast, all animals with hepatic insufficiency have too few functioning hepatocytes, but they may not have biochemical evidence of active hepatocyte damage. To emphasize the last concept, would the serum activity of liver-derived enzymes (e.g., alanine transaminase, ALT) increase if one removed an animal's liver?

C. *Cholestasis* (from the Greek *chole*, bile, and *stasis*, stoppage), is a state of impaired bile flow resulting from any of several insults, most often inflammatory, neoplastic, or toxic. There may be little or no accompanying hepatocellular damage or hepatic insufficiency, but cholestasis promotes hepatic damage and can alter intestinal function. Bile flow impairment may be obstructive or functional.

1. Bile is formed by hepatocytes and bile duct epithelial cells (cholangiocytes) through three processes.[1–3]
   a. In a BA-dependent process, active secretion of BAs from hepatocytes (mostly periportal) into canaliculi via bile salt export pumps (BSEPs) produces an osmotic gradient that increases H_2O excretion, in part through aquaporins, and that increases bile flow. Increased secretion of BAs increases bile formation.
   b. In BA-independent processes, active transport of Na^+, glutathione, and HCO_3^- osmotically promotes H_2O excretion and bile formation. Glutathione (a tripeptide) excretion is a major determinant of BA-independent bile formation, and membrane GGT contributes to metabolizing the glutathione to amino acids, which are resorbed by membrane transporters, allowing more glutathione to be produced and excreted. Glutathione, like Bc, is transported by multidrug resistance-associated protein 2 (MRP2).
   c. Cholangiocytes of larger bile ducts respond to secretin (a hormone produced in the duodenum) by activating Cl^- channels and membrane transporters that result in fluid and HCO_3^- secretion, thus alkalinizing and increasing the volume of bile.
2. In canaliculi, BAs are quickly incorporated into micelles with phospholipids and cholesterol, thus protecting cells from toxic effects of BAs and keeping cholesterol in solution. Canalicular fluid flow, promoted by contractions of pericanalicular actin-myosin filaments in hepatocytes, carries BAs downstream, further limiting local BA toxicity.
3. Components of hepatic bile include H_2O, BAs (at least 50 % of bile solids), Bc, lecithin, cholesterol, phosphatidylcholine, fatty acids, electrolytes (including Na^+, K^+, Ca^{2+}, Cl^-, and HCO_3^-), IgA, glutathione, and a variety of H_2O-soluble wastes. In the biliary ducts and gallbladder, epithelial cells secrete mucin, and BA-rich bile is formed by resorption of H_2O and some electrolytes.
4. The differences between BAs and bilirubin are important to recognize.
   a. Bu is a product of heme degradation, whereas BAs are products of cholesterol degradation. Heme and cholesterol do not share physiologic pathways.
   b. Bilirubin (Bc and Bu) and BAs enter hepatocytes through sinusoidal membranes but not via a single membrane transport system. Bc and BAs are secreted from

hepatocytes through canalicular membranes by shared and distinct membrane transport systems. Therefore, pathologic states may selectively interfere with transport across membranes, impairing uptake or secretion of one analyte more than the other.

c. Pathologic states that damage hepatocyte membranes can impair both bilirubin and BA excretion, and the accumulation of BAs in hepatocytes may cause hepatocyte damage that interferes with cell functions, including bilirubin metabolism and excretion.

5. *Obstructive cholestasis* refers to a partial or complete mechanical impediment to the flow of bile through the biliary tree, thus causing upstream biliary congestion and reduced bile excretion. Associated findings are distended bile ducts and microscopically detected bile plugs and lakes.
   a. Hepatic obstructive cholestasis occurs when the lesion impairing bile flow is within the liver. Examples include compression of biliary canaliculi by lipid-laden hepatocytes in hepatic lipidosis or by a neoplasm expanding within the liver.
   b. Extrahepatic (posthepatic) obstructive cholestasis occurs when the lesion impairing bile flow is outside the liver. Examples include blockage of the common bile duct by gallstones or pancreatitis-associated compression.
6. *Functional cholestasis* refers to reduced bile production and flow because of impaired hepatocellular secretion of bile components, principally BAs. There is no obstructive lesion.
   a. As the osmotic effects of secreted BAs largely control bile volume, decreased BA secretion reduces the volume of bile produced by the biliary system and thus reduces bile flow (less bile volume per day).
   b. Functional cholestasis is most often recognized in the context of *sepsis-associated cholestasis*, in which case endotoxin or inflammatory cytokines inhibit hepatocellular secretion of BA (see Bilirubin concentration III.G.3 and Bile Acid (BA) Concentration III.B.2.d).
7. Although cholestasis is most often recognized by hyperbilirubinemia or the icteric appearance of patients or patient samples caused by bilirubin, bilirubin is only a very small component of bile and contributes little to bile formation or bile volume. Cholestasis is associated with decreased excretion of other less-apparent substances, such as BAs and cholesterol, and there are other biochemical markers to support cholestasis (see Chapter 12 for ALP and GGT).

SUMMARY OF ABNORMAL LABORATORY TEST RESULTS ASSOCIATED WITH HEPATIC DISEASE

Many laboratory tests can yield abnormal results because of hepatic disease (Tables 13.3–13.5). These tests and results are largely nonspecific, but they are noted here because they should be considered together when evaluating patients for hepatic insufficiency or other hepatic disorders. Many of these abnormalities are the result of alterations in physiologic hepatic functions involving (i) protein metabolism (synthesis of albumin and most plasma globulins other than immunoglobulins), (ii) carbohydrate metabolism (gluconeogenesis), (iii) lipid metabolism (production and degradation of lipoproteins), (iv) storage of compounds and elements (glycogen, triglyceride, iron, or copper), (v) detoxification of endogenous and exogenous compounds (conversion of NH_4^+ to urea), (vi) actions of the mononuclear phagocyte system (responses to organisms and endotoxins), and (vii) excretion (conjugation of bilirubin, degradation of cholesterol to BAs, bile formation, and bile excretion). See other chapters for detailed information of abnormalities not discussed here.

Table 13.3. Serum or plasma chemistry test abnormalities that suggest or indicate hepatobiliary disease

Abnormality	Hepatobiliary lesion(s)	Pathogenesis of abnormality[a]
↑ ALT, AST, ID/SDH, LD, or GMD	Hepatocellular injury	Release of hepatocyte cytoplasmic enzymes due to blebbing or necrosis (Ch. 12)
↑ [Bilirubin]	Cholestasis	↓ Biliary excretion of bilirubin
↑ [Cholesterol]	Cholestasis (obstructive)	↓ Biliary cholesterol excretion (Ch. 16) ↓ Lipoprotein clearance by hepatocytes (Ch. 16) ↑ Production of cholesterol (Ch. 16)
↑ ALP activity	Cholestasis	↑ Production of L-ALP (Ch. 12)
↑ GGT activity	Cholestasis	↑ Production of GGT (Ch. 12)
	Biliary hyperplasia	↑ Production of GGT (Ch. 12)
	Possibly hepatocellular injury (horses, adult cattle)	GGT release from damaged hepatocytes (Ch. 12)
↓ [Albumin]	↓ Functional mass[b]	↓ Production of albumin (Ch. 7)
↓ [Cholesterol]	↓ Functional mass	↓ Production of cholesterol (Ch. 16)
↓ [Glucose]	↓ Functional mass	↓ Production of glucose (Ch. 14)
↓ [Total protein]	↓ Functional mass	↓ Production of albumin and globulins other than immunoglobulins (Ch. 7)
↓ [UN]	↓ Functional mass	↓ Production of urea (Ch. 8)
Lipemia (gross)	↓ Functional mass	↓ Clearance of lipoproteins (Ch. 16)
↑ [Glucose] (rare)	Cirrhosis or other hepatopathy related to hepatocutaneous syndrome	Hyperglucagonemia or ↑ gluconeogenesis (Ch. 14)
↑ [NH_4^+]	↓ Functional mass	↓ Fixation of NH_4^+ into urea (Ch. 13)
	Portosystemic shunts	↓ NH_4^+ delivery to hepatocytes (Ch. 13)
↑ [BAs]	↓ Functional mass	↓ Clearance of BAs from portal blood (Ch. 13)
	Portosystemic shunts	↓ BA delivery to hepatocytes (Ch. 13)
	Cholestasis	↓ Biliary excretion of BAs (Ch. 13)
↑ [Uric acid]	↓ Functional mass	↓ Conversion of uric acid to allantoin (Ch.8)

[a] See more complete explanations in cited chapters for each particular analyte.
[b] Decreased functional mass results from diffuse hepatocyte damage, destruction, atrophy, or hypoplasia.
L-ALP, liver isoform of ALP; UN, urea nitrogen

Table 13.4. Hemostasis and CBC test abnormalities that support hepatobiliary disease

Abnormality	Hepatobiliary lesion(s)	Pathogenesis of abnormality[a]
Hemostasis testing (Ch. 5)		
Prolonged PT or PTT	↓ Functional mass,[b] cholestasis	↓ Production of coagulation factors ↓ Clearance of inhibitors of coagulation factors such as FDPs ↓ Carboxylation of vitamin K–dependent factors due to ↓ intestinal absorption of vitamin K
↓ [Fibrinogen]	↓ Functional mass	↓ Production of fibrinogen (Ch. 7)
↑ [FDP]	↓ Functional mass	↓ Hepatic clearance of FDPs
↑ [D-dimer]	↓ Functional mass	↓ Hepatic clearance of D-dimers
Complete Blood Count (CBC) (Ch. 3)		
Microcytosis	↓ Functional mass, portosystemic shunt	Possibly ↓ transferrin production and thus ↓ delivery of iron to RBC precursors
Codocytosis	↓ Functional mass	Altered lipid composition of RBC membrane
Acanthocytosis	Neoplasms, hepatitis, ↓ functional mass	Altered lipid composition of RBC membrane in people; unknown in domestic mammals
Anemia	Hepatitis (chronic)	Anemia of inflammatory disease
	↓ Functional mass	Possibly ↓ erythropoietin or abnormal protein or amino acid metabolism

[a] See more complete explanations in cited chapters for each particular analyte.
[b] Decreased functional mass results from diffuse hepatocyte damage, destruction, atrophy, or hypoplasia.
PT, prothrombin time; PTT, partial thromboplastin time; FDPs, fibrin(ogen) degradation products

BILIRUBIN CONCENTRATION

I. Physiologic processes (Figure 13.1)
   A. In plasma, there are three major fractions of Bt; these are: Bu/Alb, Bc (mono- and diconjugated forms), and Bδ. Bu/Alb is dominant in the absence of disease; the equilibrium of the Bu interaction with albumin is such that only a very small amount of unconjugated bilirubin (estimated to be 1/50,000th) circulates unbound as *free bilirubin*, which may be important in bilirubin toxicity.[6] Free Bu readily passes through lipid bilayer membranes and can cross the blood–brain barrier. Bu is constantly produced from the degradation of heme from senescent RBCs and heme-containing proteins. In health, Bu and Bc are removed from plasma by either liver (Bu and Bc) or kidneys (Bc). The spontaneous and covalent binding of bilirubin glucuronide isomers (Bc) to albumin forms Bδ (Bc-Alb), which, once formed, has the circulating half-life of albumin (~8–20 d).[7] Bc and Bδ concentrations are negligible in health.
   B. Small amounts of bilirubin are commonly found in the urine of healthy dogs, but not of other common domestic species (see Bilirubinuria, Chapter 8).
   C. Healthy horses have greater serum [Bt] than dogs, cats, cattle, alpacas, and llamas: about 0.7–2.0 mg/dL (mostly Bu/Alb) in horses versus < 0.5 mg/dL in the other species.

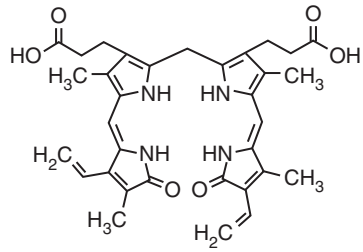
Table 13.5. Urinalysis, fecal, and peritoneal fluid abnormalities that support hepatobiliary disease

Pathologic finding	Hepatobiliary lesion(s)	Pathogenesis of abnormality[a]
Urinalysis results (Ch. 8)		
Ammonium (bi)urate crystalluria[b]	↓ Functional mass, urea cycle defect	↓ Fixing of NH_4^+ into urea and ↓ conversion of uric acid to allantoin
Bilirubinuria[b]	Cholestasis, obstructive or functional	↓ Biliary excretion of bilirubin
Hyposthenuria or isosthenuria[b]	↓ Functional mass	↓ Renal medullary tonicity due to ↓ urea; ↑ NH_4^+ excretion may inhibit renal concentrating mechanism
Urate crystalluria[b]	↓ Functional mass	↓ Conversion of uric acid to allantoin
Fecal exam results		
Steatorrhea	Cholestasis	Defective lipid digestion from ↓ BA delivery to intestine
Peritoneal fluid analysis (Ch. 19)		
Transudate	↓ Functional mass; presinusoidal or sinusoidal hypertension	↑ Na^+ and H_2O retention, ↓ plasma oncotic pressure, ↑ plasma hydraulic pressure, ↓ lymph drainage

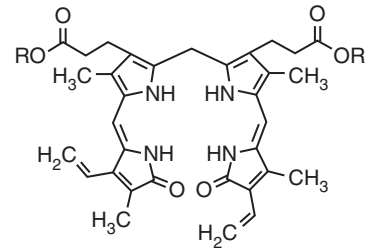
[a] See more complete explanations in the chapters for each particular analyte.
[b] Finding may occur in clinically healthy animals, e.g., ammonium (bi)urate crystalluria in Dalmatians, urate crystalluria in English bulldogs, bilirubinuria in dogs, and hyposthenuria or isosthenuria as a physiologic response to excess H_2O intake.

II. Analytical concepts

A. Unit conversion (used for both Bu and Bc, because the concentration of only the bilirubin part of Bc is of interest): mg/dL × 17.10 = μmol/L (SI unit)[8]



Bilirubin



Diconjugated Bilirubin

B. Methods

1. Most clinical assays for serum or plasma bilirubin have employed some version of the *diazo method*, in which diazotized sulfanilic acid or other diazo reagent reacts with the tetrapyrrole bilirubin (but not biliverdin) at the central carbon to form two azodipyrroles called azobilirubin. The color intensity of azobilirubin is proportional to the concentration of bilirubin in the sample and is measured by absorbance photometry. Variations of the diazo method enable detection of different bilirubin fractions.

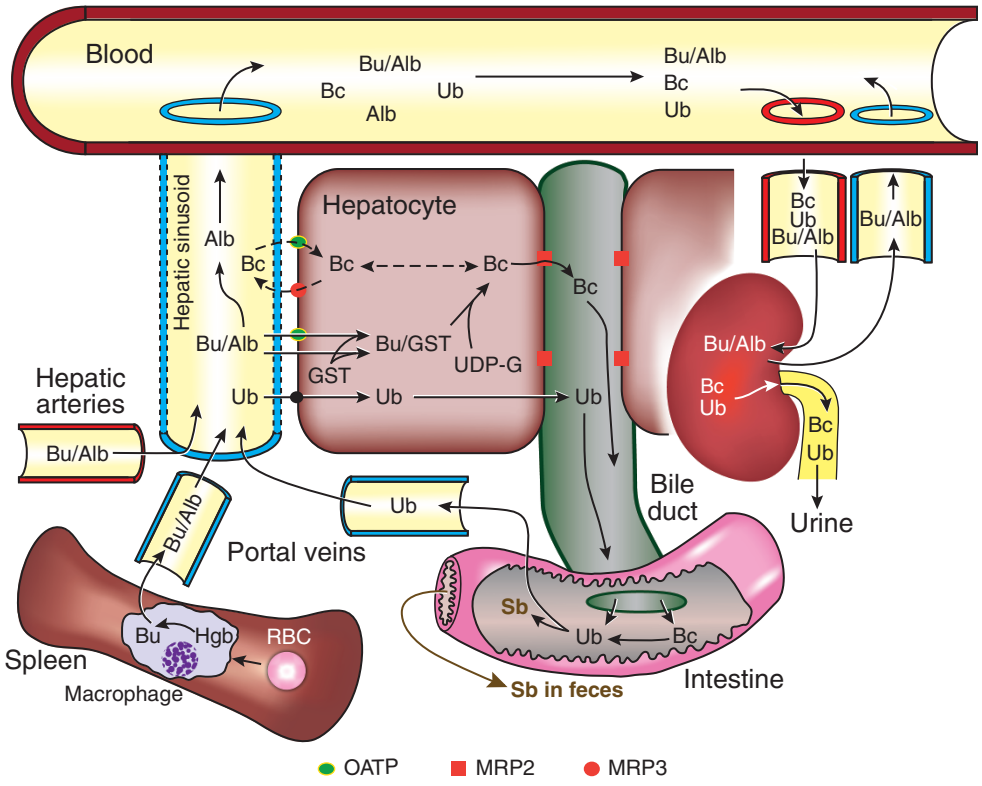


Figure 13.1. Physiologic processes involving bilirubin.

- In health, RBC destruction within macrophages (e.g, of the spleen, liver, or bone marrow) is followed by the degradation of heme and its conversion to Bu. Clinically insignificant amounts of Bu, about 25 % of [Bu], are formed from heme degradation associated with ineffective erythropoiesis and degradation of other heme-containing molecules (catalase, peroxidase, and cytochromes). As Bu leaves a macrophage, it forms a strong but noncovalent association with albumin (Alb) and remains associated with Alb until uptake by hepatocytes. Bu is relatively H_2O insoluble prior to binding to Alb.
- After Bu enters the liver and its protein-permeable sinusoids, it enters hepatocytes without albumin and binds to cytosolic proteins including glutathione S-transferases (GSTs, also called ligandins) and fatty acid–binding protein (not shown). Bu probably enters hepatocytes by passive diffusion as well as via incompletely defined organic anion transporters that likely include organic anion-transporting polypeptides (OATPs);[4,5] the binding proteins in hepatocytes enhance the process by reducing diffusion of Bu back to the sinusoidal plasma.
- Within hepatocytes, Bu is conjugated with glucuronide (also glucose in horses) to form bilirubin monoglucuronide or bilirubin diglucuronide, collectively called Bc. Conjugation disrupts intramolecular hydrogen bonds, thereby opening the molecule and making it more H_2O soluble.
- Bc is transported from hepatocytes into canaliculi (the rate-limiting step in bilirubin excretion) by energy-dependent transport via multidrug resistance-associated protein 2 (MRP2), which also transports some BAs. Prior to secretion, Bc can move from hepatocytes to sinusoidal blood via multidrug resistance-associated protein 3 (MRP3) and back to hepatocytes via sinusoidal transmembrane organic anion-transporting polypeptides (OATPs).
- Bc in bile enters the intestine and is degraded to urobilinogen (colorless). Urobilinogen can be passively absorbed in the intestine and then either enter hepatocytes for excretion in bile or bypass the liver and be excreted in urine. Urobilinogen can also be degraded to stercobilinogen (dark brown) and excreted in feces.
- Bc that passes into blood without reuptake can pass through the glomerular filtration barrier and be excreted in the urine. Because Alb does not pass through the glomerular filtration barrier of most healthy mammals, Bu/Alb does not enter the urine in those animals.

Bu/Alb, Bu associated with albumin; Sb, stercobilinogen; Ub, urobilinogen; UDP-G, uridine diphosphoglucuronide

a. Assays that incorporate reaction accelerants (e.g., caffeine and sodium benzoate, dyphilline), which help dissociate Bu from albumin, detect all bilirubin fractions, thus yielding [Bt].
b. Assays without accelerants detect primarily Bc and Bδ, yielding [Bd], the concentration of bilirubin fractions measured directly with this *direct bilirubin reaction* (i.e., without use of agents to dissociate Bu from albumin). Small amounts of Bu are measured as well, causing overestimation of [Bd], especially when [Bt] < 1 mg/dL.[6] Therefore, [Bd] does not exactly equate to [Bc]. The [Bδ] cannot be determined by this method.
c. The calculated difference between measured [Bt] and measured [Bd] is the concentration of the remaining bilirubin, which is mostly Bu and is referred to as [Bi] because it is determined indirectly rather than by direct measurement: [Bi] = [Bt] – [Bd]. [Bi] does not exactly equate to [Bu].
d. Most of these methods are "wet" chemical assays, but the diazo reaction with accelerator has also been applied to "dry" chemical assays for [Bt] using reagent slides and reflectance photometry (e.g., IDEXX Catalyst, Kodak or Vitros instruments).

2. The dual-wavelength reflectance spectrophotometry slide method for Bc and Bu (Kodak or Vitros instruments) relies on changes in spectral properties of bilirubin fractions that occur when they bind to a cationic polymer, the mordant that fixes bilirubin molecules to the slide.[9–11]
a. When bound to the mordant, the mono- and diconjugated bilirubins can be detected together as Bc, but Bc and Bu can also be detected separately because they have different spectral properties; Bδ is not detected because it is excluded by the slide technology.
b. When results for [Bc] and [Bu] are combined with the [Bt] from the diazo reaction slide, [Bδ] can be calculated because [Bt] includes [Bδ], but [Bδ] is not included in the other assays. [Bδ] = [Bt] – ([Bu] + [Bc]).
c. [Bd] can also be calculated: [Bd] = [Bc] + [Bδ].

3. Enzymatic bilirubin assays employ bilirubin oxidase to convert bilirubin to biliverdin, and the decrease in absorbance related to bilirubin is measured. Alterations in pH allow measurement of various bilirubin fractions.
4. The nonenzymatic vanadate oxidase method similarly oxidizes bilirubin to biliverdin, but via the oxidative properties of vanadate, an oxoanion of vanadium. In the presence of a detergent, all bilirubin is oxidized to biliverdin, so [Bt] is measured. In the absence of detergent, only [Bd] is measured.[12]
5. High performance liquid chromatography can be used to measure proportions of various bilirubin fractions; from these and a [Bt] determined chemically, fraction concentrations can be calculated.

C. Sample handling and interference issues
1. Hgb interferes with azo reactions and produces falsely low [Bt] with routine wet chemistry methods. However, in the thin-layer reagent slides, Hgb falsely increases [Bt] and [Bc] while falsely decreasing [Bu].
2. Ultraviolet and blue light degrade bilirubin, in vitro and in vivo. Isomerization yields some products that are not measured by routine assays, and hydrolysis converts Bc to Bu. Therefore, in vitro exposure of blood or urine to light may decrease [Bt] and [Bc], particularly the latter.
a. In direct sunlight, [Bt] may decrease by up to 50 % in 1 h.
b. In artificial light and room temperature of laboratory settings, small but clinically significant changes in [Bt] and [Bd] may occur in 2–24 h with human serum or

heparinized plasma samples.[13,14] [Bt] and [Bd] decreased more rapidly in canine serum and urine samples under similar laboratory conditions (unpublished data, M.A.S.). Such preanalytical errors are not expected with routine processing and testing within 2 h of collection.

3. Rarely, immunoglobulins may precipitate after the addition of acidic bilirubin reagents used in certain assays, and this precipitant interferes with light transmission and causes a falsely increased [Bt].[15] The interference occurs primarily when there is an increased γ-globulin concentration because of a paraproteinemia. A clearly increased [Bt] (e.g., > 5 mg/dL) can be easily recognized as a false value when the serum is visually not icteric. In one case, the [Bt] was "measured" to be > 20 mg/dL, but the sample was not icteric (unpublished observation, S.L.S.). Minor interferences, however, may not be detected via this comparison.
4. Using thin-layer slide assays, the sum of [Bc] and [Bu] may exceed the measured [Bt] (especially in horses). It has not been established whether the [Bt] is falsely decreased or whether the sum of [Bc] and [Bu] is falsely increased.

III. Hyperbilirubinemia (Table 13.6)

A. Mechanistically, hyperbilirubinemia occurs when the rate of Bu production exceeds the rate of Bu uptake by hepatocytes, or when the rate of Bc formation in hepatocytes exceeds the rate of Bc excretion in bile. It may persist after resolution of the underlying problem because of the long half-life of Bδ.

B. Causes of hyperbilirubinemia can be classified by mechanism as outlined below or by the relationship of the underlying problem to the liver, i.e., as prehepatic, hepatic, or posthepatic hyperbilirubinemia or icterus. Use of the latter classification requires clarity of the terms. As previously defined for this book, *hepatic* refers to all parts of the liver (not just hepatocytes) and does not include the common bile duct or gall bladder, which are posthepatic. Although some authors consider any event or lesion occurring after bilirubin conjugation to be posthepatic, whether it occurs in the liver or not, those occurring in the liver (e.g., Bc secretion, compression of hepatic bile ducts) are considered hepatic in this book. When prehepatic factors lead to hyperbilirubinemia through impairment of bilirubin processing by hepatocytes, the hyperbilirubinemia is classified herein as hepatic because it is ultimately caused by a disturbance in hepatocyte function.

C. General interpretive guidelines for moderate or marked hyperbilirubinemia in species other than horses: Hyperbilirubinemia without evidence of hemolysis (not always clear) supports hepatic or posthepatic cholestatic disease. Hyperbilirubinemia with a clear predominance of Bc also suggests cholestatic disease. Hyperbilirubinemia with a clear predominance of Bu supports hemolysis or a rare genetic defect in bilirubin conjugation. Hyperbilirubinemia with similar concentrations of Bu and Bc suggests a component of hepatocellular disease. Hepatocellular, hepatobiliary, and hemolytic disease can be concurrent.

D. Increased Bu production

1. Hemolytic hyperbilirubinemia or icterus (prehepatic) (Figure 3.20)
   a. Hemolytic disorders that may cause hyperbilirubinemia and icterus are listed in Table 3.12. Animals with acute intravascular hemolytic disorders are less likely than those with primarily extravascular hemolysis to be icteric initially, but will typically develop icterus within 2–3 d. If the rate of extravascular hemolysis is low to moderate, the hepatobiliary system may be able to eliminate Bu so it does not accumulate in plasma.

b. Pathogenesis: As described in Chapter 3, an animal that has a hemolytic anemia may develop icterus if the rate of Bu formation exceeds the hepatobiliary system's capacity for Bu uptake or Bc excretion (Figure 3.20).
c. Results of serum or plasma bilirubin profiles (Table 13.7)
   (1) [Bt] may be mildly to markedly increased.
   (2) Initially, the [Bu] is much greater than the [Bc]. If hemolysis persists, then [Bu] may approximate [Bc] as [Bc] increases. When [Bu] > [Bc], it supports that the icterus is directly related to extravascular hemolysis.
   (3) It has been written that bilirubin fractions do not help differentiate hemolytic and hepatobiliary icterus because [Bc] is always greater than [Bu].[16] This conclusion, based in part on findings in eight dogs with concurrent hemolytic anemia and liver disease,[17] is not consistent with the authors' experiences and with expected findings in people.[6]
   (4) [Bu] is usually greater than [Bc] in dogs and cats with hemolytic anemias, but it is not always greater. However, if [Bc] > [Bu] and clinical data indicate a hemolytic anemia, consideration should be given to concurrent primary hepatobiliary disease or cholestasis secondary to hypoxic liver damage.

Table 13.6. Disorders and conditions that cause hyperbilirubinemia

Increased Bu production (prehepatic)[a]
Hemolytic disorders,* especially extravascular hemolysis (Tables 3.12 and 3.14)
Decreased Bu uptake by hepatocytes (hepatic)[a]
Fasting or anorexia* (especially in horses)
↓ Functional mass[b] (diffuse hepatocellular disease; see the text for examples)
Decreased Bu conjugation (hepatic)[a]
↓ Functional mass[b] (diffuse hepatocellular disease; see the text for examples)
Decreased Bc excretion in bile (hepatic or posthepatic)[a]
Obstructive cholestasis
Hepatic[a] cholestasis:* lipidosis, diabetes mellitus, steroid hepatopathy, hepatic lymphoma or other neoplasms, histoplasmosis, cytauxzoonosis, flukes affecting liver bile ducts, cirrhosis, cholangitis, cholangiohepatitis, periportal hepatitis, pyrrolizidine alkaloid toxicosis, sporodesmin toxicosis, alsike clover (*Trifolium hybridum*) toxicosis
Posthepatic[a] cholestasis:* cholangitis, bile duct carcinoma and other neoplasms or cysts, liver flukes or rarely helminths in common bile duct, cholelithiasis, cholecystitis, congenital hepatobiliary malformations, pancreatitis (acute inflammation or fibrosis), pancreatic carcinoma, intestinal foreign body or neoplasm
Functional cholestasis (sepsis-associated cholestasis) (hepatic)[a]
Persistence of Bδ in plasma (prehepatic, hepatic, posthepatic)[a]

* A relatively common disorder, condition, or mechanism
[a] Prehepatic, caused by increased bilirubin production; hepatic, caused by factors involving hepatocytes and biliary ducts within the liver; posthepatic, caused by factors affecting the gallbladder or common bile duct
[b] By itself, hepatic insufficiency (cirrhosis) is not expected to cause hyperbilirubinemia.
Notes: Lists of specific disorders or conditions are not complete but are provided to give examples. Depending on the assay method, hemolyzed samples may cause either positive or negative interference with bilirubin assays. False decreases in [Bt] and [Bc] may occur when a sample is excessively exposed to light, and false increases in [Bt] may rarely occur with paraproteinemia (see the text).

Table 13.7. Typical bilirubin profiles in common pathophysiologic states that cause hyperbilirubinemia

[Bd] or [Bc]	[Bi] or [Bu]	[Bδ]	[Bd] vs [Bi]	Pathophysiologic state
WRI–↑[ab]	↑↑	WRI–↑	[Bd] < [Bi]	Hemolytic disorders, fasting hyperbilirubinemia (horses)
↑↑	WRI–↑	WRI–↑↑	[Bd] > [Bi]	Obstructive or functional cholestasis[c]
↑↑	↑–↑↑	WRI–↑↑	Variable	Hepatocellular dysfunction and concurrent cholestasis

[a] The number of arrows for the bilirubin fractions in the table indicates relative concentrations; illustrated using a moderately increased [Bt] for all states.
[b] Transport of Bc from hepatocytes to canaliculi is considered the rate-limiting step in hepatic bilirubin excretion. Thus, when there is increased Bu and Bc formation in hemolytic disorders, Bc may accumulate in hepatocytes and then be "regurgitated" (transported) to blood.
[c] In horses, the [Bu] may be greater than the [Bc] because of concurrent inappetence or anorexia.
Note: Depending on the assay method, hemolyzed samples may cause either positive or negative interference with bilirubin assays. See the text for other interferences.

d. Other common laboratory findings with hemolytic hyperbilirubinemia
   (1) Moderate to marked anemia, regenerative if of sufficient duration
   (2) Bilirubinuria if sufficient Bc escapes from hepatocytes or, with intravascular hemolysis in dogs, if there is sufficient renal tubular heme degradation and bilirubin excretion into urine (Note: Healthy dogs with concentrated urine may have mild to moderate bilirubinuria, as described in section IV.C.)
   (3) Associated RBC poikilocytes, inclusions, or organisms
   (4) Hemoglobinuria and hemoglobinemia if there is sufficient intravascular hemolysis

2. Internal hemorrhage, destruction of RBC precursors, and degradation of other heme proteins (myoglobin, cytochromes, and peroxidases) can increase the generation of Bu and contribute to increased [Bu] and [Bt], but the rate of Bu formation due to any of those processes alone is generally unlikely to increase [Bt] above the URL.

E. Decreased Bu uptake by hepatocytes (hepatic)
1. Fasting hyperbilirubinemia
   a. Disorders
      (1) Horses that are off feed (feed withheld or anorexic) commonly develop moderate hyperbilirubinemia (increased [Bu]).[18] The hyperbilirubinemia can be detected as early as 12–15 h after feed is withheld and usually reaches a steady concentration in 2–3 d.
      (2) Sick cattle with anorexia and rumen stasis may have hyperbilirubinemia (increased [Bu] not associated with hemolytic or hepatic disease).[19] Fasting healthy cattle also develop mild hyperbilirubinemia ([Bt] < 1.4 mg/dL).[20]
      (3) Lack of colostrum intake was considered a contributing factor to icterus in veal calves.[21]
   b. Pathogenesis in horses: The fasting state leads to increased mobilization of fat (lipolysis in adipocytes) and thus increased [fatty acids] in the blood. The fatty acids interfere with Bu uptake by hepatocytes, and thus plasma [Bu] increases.[20]

(1) The interference may be competitive because fatty acids and Bu bind to the same cytoplasmic receptor proteins: fatty acid–binding protein and glutathione S-transferase B (ligandin).[22,23]
(2) Experimental data clearly indicate that fasting reduces the hepatic clearance rate of bilirubin and that the reduction is greater than the reduction in the hepatic clearance of BAs.[18]
(3) This is classified herein as hepatic hyperbilirubinemia or icterus because hepatocyte Bu uptake is impaired (see III.B above); however, the initiating factor for the impairment is prehepatic.

c. Comments on species differences: Horses might be more prone to this form of icterus because they use primarily glucose (> 60 %) to conjugate bilirubin.[24] Fasting may make less glucose available in hepatocytes for Bu conjugation. Administration of glucose to horses with fasting hyperbilirubinemia lowered plasma concentrations of bilirubin and fatty acids.[25] It was not established whether the lower [Bt] was due to availability of glucose or to a reduced [fatty acids].
d. Similar interferences in bilirubin excretion likely occur in other animals, but changes in [Bt] are minimal. Anorectic cats occasionally have slight hyperbilirubinemias (increased [Bu]), and thus it is easy to speculate that fatty acids are contributing to defective Bu clearance. Decreased feed intake did not cause hyperbilirubinemia in eight llamas, five of which developed hepatic lipidosis.[26]
e. Results of a serum or plasma bilirubin profile (Table 13.7)
(1) In horses, the [Bt] is typically < 8 mg/dL, and nearly all is [Bu] or [Bi].
(2) Minor increases in [Bc] or [Bd] may occur because Bu and Bc compete for uptake by hepatocytes or because of incomplete analytical separation of bilirubin fractions.
f. Other laboratory findings with fasting hyperbilirubinemia
(1) In horses, several days of fasting or anorexia do not produce abnormalities in other routine laboratory tests. If plasma or serum AST, ID/SDH, LD, or GMD activities are increased, hepatobiliary disease should be considered, and hemolytic anemia should be considered if there is concurrent anemia or other signs of hemolytic disease.
(2) In cattle or horses with more prolonged or severe negative energy balance, hepatic lipidosis may develop. Accumulation of lipid in hepatocytes may enlarge them and cause biliary obstruction and hepatocellular damage. These may be reflected in hyperbilirubinemia (including [Bd] or [Bc]) and increased activities of GGT, ALP, AST, ID/SDH, LD, or GMD (see Chapter 12).

2. Decreased functional hepatic mass (Table 13.2): A marked reduction in functional hepatocytes can result in decreased Bu uptake by hepatocytes, decreased Bu conjugation, and decreased Bc excretion to bile, potentially increasing [Bt], [Bu], and [Bc]. A reduction in the hepatocyte transport capacity to recycle the Bc that is normally exported to blood (see OATPs in Figures 13.1 and 13.2) could also promote an increased [Bc] without cholestatic disease. However, decreased hepatic functional mass does not necessarily result in hyperbilirubinemia.
3. Hereditary deficiencies in the hepatic uptake of Bu may cause hyperbilirubinemia and have been recognized in Southdown sheep[27] and Corriedale sheep.[28]

F. Decreased Bu conjugation (hepatic)
1. As just mentioned in the preceding Section E.2, a marked reduction in hepatic functional mass (hepatocytes) would reduce any aspect of hepatocellular bilirubin processing, including Bu conjugation.

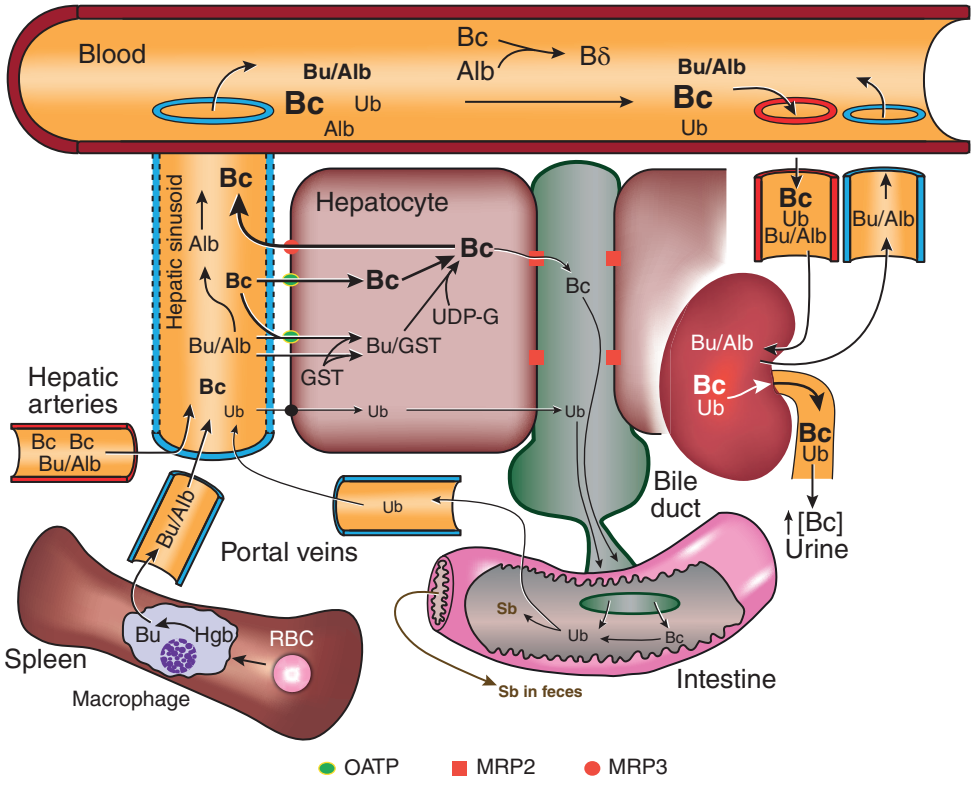


Figure 13.2. Obstructive cholestatic icterus: Lesions of bile canaliculi or bile ducts (hepatic or posthepatic) inhibit bile flow, and thus less or no Bc is excreted to the intestine. With established cholestasis, there is less excretion of Bc from hepatocytes to canaliculi (via downregulation of MRP2) and increased pumping of Bc from hepatocytes to sinusoids (via upregulation of MRP3); the latter movement can explain the concept of cholestatic Bc "regurgitation" to hepatic sinusoids. Increased [Bc] in sinusoidal blood competes with Bu for hepatocyte uptake by organic anion transporters, likely including organic anion transporting polypeptides (OATPs), so Bu uptake is impaired and [Bu] (Bu being present as Bu/Alb) may increase. A persistently increased plasma [Bc] results in increased formation of Bδ and increased urinary excretion of Bc (bilirubinuria), which further increases the percentage of plasma bilirubin in the form of Bδ. Because Bδ is slowly cleared from plasma, [Bt] and [Bd] may decrease slowly after alleviation of biliary obstruction.
Bu/Alb, Bu associated with albumin; Bδ, Bc covalently bound to albumin; MRP, multidrug resistance-associated protein; Sb, stercobilinogen; Ub, urobilinogen; UDP-G, uridine diphosphoglucuronide

2. Hereditary deficiencies of enzymes that catalyze conjugation reactions occur in primates and laboratory animals but have not been confirmed in common domestic mammals. Persistent hyperbilirubinemia in an otherwise healthy thoroughbred horse was probably caused by a defective conjugation pathway, but the exact defect was not established.[29] The [Bt] ranged from 9.4 to 12.7 mg/dL during a 2-yr evaluation, and about 90 % of the bilirubin was Bu.

G. Decreased Bc excretion (hepatic or posthepatic)

1. *Obstructive cholestasis* is caused by a pathologic state that mechanically impairs bile flow through bile canaliculi or hepatic bile ducts (hepatic obstructive cholestasis) or common bile duct (posthepatic obstructive cholestasis). Lesions that impair bile flow include

hepatocellular swelling that compresses canaliculi, periportal lesions that compress bile ducts, infections and other processes that damage bile ducts, and blockage of bile ducts by stones, parasites, or neoplasms.

2. Although bilirubin is an important diagnostic marker of cholestasis, Bc is a very small component of bile and contributes little to formation of bile or bile volume. Importantly, when there is obstructive biliary disease causing decreased bile flow and decreased Bc excretion, BA excretion will also be decreased and serum [BAs] is typically increased.
   a. Pathogenesis (Figure 13.2)
      (1) With biliary obstruction, hepatocytes transport less Bc into canaliculi and more into sinusoidal blood. There might also be increased leakage from damaged canaliculi, tight junctions around them, or bile ductules.
      (2) Increased [Bc] in sinusoidal (and systemic) blood may competitively impair hepatocyte uptake of Bu, leading to increased [Bu]. Hepatocyte damage may also decrease Bu uptake, and inhibition of Bu conjugation secondary to increased hepatocyte Bc may also contribute to increased [Bu].[6] So, with time, increased [Bc] leads to increased [Bu], but the [Bc] is expected to remain greater than the [Bu].
   b. Results of a serum or plasma bilirubin profile (Table 13.7)
      (1) The [Bt] may be mildly to extremely increased.
      (2) Typically, [Bc] > [Bu] ([Bd] > [Bi]) except in horses and cattle, and concurrent reduction in hepatic functional mass could complicate patterns by decreasing uptake and conjugation of Bu.
         (a) In horses with cholestasis, the [Bd] is often < 50 % of [Bt]. Increased [Bd] in horses strongly suggests cholestasis even if there is an increased [Bi].[30]
         (b) In cattle with cholestasis, the [Bd] may be greater than or less than the [Bi].
   c. Other laboratory findings with obstructive cholestasis
      (1) Increased serum activities of enzymes associated with cholestasis (ALP and GGT)
         (a) ALP and GGT activities may be increased in dogs before icterus develops.
         (b) ALP and GGT activities typically are not increased in cats until there is marked obstructive icterus.
         (c) Biliary obstruction is uncommon in horses and cattle. When present, ALP and GGT activities may be substantially increased.
      (2) Serum or plasma markers of hepatocellular injury (ALT, AST, LD, ID/SDH, and GMD activities, see Chapter 12) may be increased because of concurrent hepatocyte damage.
      (3) Hypercholesterolemia occurs in some cholestatic conditions and can be marked but is not a consistent finding (see Chapter 16).
3. *Functional cholestasis* (*sepsis-associated cholestasis*) is nonmechanical impairment of Bc, BA, and H_2O excretion from hepatocytes. It was reported in dogs with *Escherichia coli* and *Staphylococcus intermedius* infections causing pneumonia, peritonitis, urinary tract infection, endocarditis, and soft tissue infection.[31] It has been more commonly recognized in human patients than in domestic mammals.
   a. Pathogenesis[32–34]
      (1) Endotoxin and other bacterial products increase expression of proinflammatory cytokines (e.g., tumor necrosis factor α, interleukins 1β and 6) by Kupffer cells, hepatocytes, and other cells, and together decrease expression of BA transport pumps (Na-dependent BA transporters and multidrug resistance-associated

protein 2), adenosine triphosphatases (ATPases), and H_2O-transport proteins (aquaporin 8) in hepatocyte canalicular membranes, thus decreasing bile production and flow. Decreased expression of multidrug resistance-associated protein 2 also decreases Bc excretion into bile.

(2) Additionally, Na-taurocholate cotransporting polypeptide and organic anion-transporting polypeptides (OATPs), which transport BAs and Bu into hepatocytes, are downregulated, whereas multidrug resistance-associated proteins (MRPs) 3 and 4, which export BAs from hepatocytes to plasma, are upregulated, thus lessening hepatocyte damage from increased BAs.

(3) Reduced bile formation and decreased Bc exporters (multidrug resistance-associated protein 2) promote hyperbilirubinemia by impairing Bc excretion. Further impairment may occur from hepatocyte damage caused by retained BAs or from microvascular damage promoted by cytokine-mediated generation of nitric oxide.

b. The serum or plasma bilirubin profile mirrors that for obstructive cholestasis (Table 13.7), but hyperbilirubinemia, mostly Bc, is typically only mild to moderate. An increased [BAs] is expected. In people, serum ALP activity is typically no more than 2–3 × URL.

c. Other expected laboratory findings with functional cholestasis

(1) Inflammatory changes in blood leukocytes (e.g., neutrophilia, neutropenia, left shift, toxic change, or lymphopenia)

(2) Alterations in concentrations of acute-phase reactants

(3) Other changes would depend on the duration and the location of the bacterial infection.

4. Hereditary deficiencies in the hepatocellular excretion of Bc may cause hyperbilirubinemia and have been recognized in Corriedale sheep. The sheep also have a defect in Bu uptake by hepatocytes.[28]

H. Persistence of Bδ in plasma

1. Because Bδ has the plasma half-life of albumin (~8 d in dogs; ~19 d in horses – see Chapter 7), an increased [Bt] may persist 1–3 wk after correction of the pathologic defect in bilirubin excretion (e.g., surgical removal of a biliary tract obstruction). It is also possible that in animals with cholestatic icterus, the half-life of Bδ may be shorter than the time required for the [Bc] to decrease if there is slow recovery from an obstructive cholestatic disease.

2. The persistent increase in [Bδ] and thus persistent increase in [Bt] is not commonly recognized because there is limited use of the dry-chemical method that enables calculation of the [Bδ] (see II.B.2.b above).

IV. Bilirubinuria

A. Excessive bilirubin formation (in hemolytic states) or impaired hepatobiliary excretion of Bc may increase plasma [Bc]. Bc easily passes through the glomerular filtration barrier and is then excreted in the urine.

B. Because of the low renal threshold for Bc, bilirubinuria may be detected before hyperbilirubinemia is detected. If hyperbilirubinemia including Bc is present, then bilirubinuria is expected.

C. As explained in Chapter 8, a positive bilirubin reaction in a urinalysis should be interpreted with knowledge of USG_{ref}, especially in dogs. Moderately concentrated urine (USG_{ref} = 1.025–1.040) of healthy dogs frequently gives a 1+ bilirubin reaction; 2+ reactions occasionally occur with more concentrated urine (USG_{ref} > 1.040).

V. Icterus index
   A. Definition: a value that represents an estimation of the yellow discoloration of plasma caused by hyperbilirubinemia
   B. Methods
      1. Potassium dichromate method[35]
         a. The icterus index is determined by comparing the color of a patient's plasma to a set of standard solutions containing potassium dichromate. The degree of icterus is recorded in units from 0 to 100. The plasma of healthy herbivores is expected to be more yellow because carotenoid pigments from plants may impart a yellow discoloration.
         b. Hemolyzed or lipemic plasma can falsely elevate the icterus index.
      2. Icterus index determination by chemistry analyzers
         a. As part of a routine screening for common interferents in chemical assays, many chemistry analyzers estimate each sample's bilirubin concentration by spectrophotometric assessment of absorbance changes that may be caused by the presence of bilirubin. The method involves concurrent assessment and correction for absorbance changes caused by Hgb or lipids, if present; however, errors may occur because of incompletely corrected effects of spectral overlap of these substances.[36] This icterus index is reported as a numerical value (e.g., 10) that corresponds to absorbance changes expected by a [Bt] of the same numeric value (e.g., 10 mg/dL). Each analyzer's index should be validated for each species of interest prior to use. If this is done and properly controlled as for other assays, unexpected mismatches between [Bt] and the icterus index may signal errors in either measurement. The numerical icterus index should not be used as a substitute for the Bt assay.
         b. Beckman Coulter (Olympus) chemistry analyzers have a similar spectrophotometric system in which absorbance readings at different wavelengths are used to assess degrees of icterus, lipemia, and hemolysis; these are reported on 1+ to 5+ scales.
         c. Plant carotenoids (e.g., β-carotene and lycopene) may interfere with these methods.
   C. Clinical laboratory personnel may subjectively grade the severity of icterus (mild, moderate, or marked) by assessing the degree of yellow to orange discoloration of plasma or serum. Serum [Bt] typically exceeds 1.5 mg/dL before visible icterus in sera or mucous membranes is detected. This depends somewhat on the species, lighting conditions, the width of the sample tube, and the observer.

BILE ACID (BA) CONCENTRATION[37]

I. Physiologic processes (Figure 13.3 and Hepatobiliary Disease and Hepatic Insufficiency, Section II.C)
   A. The term *bile acids* is commonly used to refer to a group of cholesterol-derived, conjugated, or unconjugated anionic acids and their dissociated anions. However, it is also used more specifically to refer to only the unconjugated forms that include cholic acid, chenodeoxycholic acid, deoxycholic acid, and lithocholic acid, all of which are 3α-hydroxylated bile acids. At a pH of 7.4, the dissociated anionic forms of the molecules dominate (e.g., cholate and lithocholate).
   B. When BAs are conjugated in hepatocytes (mostly to taurine or glycine), they become more fully ionized, more H_2O soluble, and better detergents. These conjugated BAs (technically, their Na^+ or K^+ salts) are referred to as *bile salts*, but "bile acids" and "bile salts" are commonly used interchangeably in the clinical setting. The most common analytical tests detect BAs and bile salts but are referred to as tests for BAs.

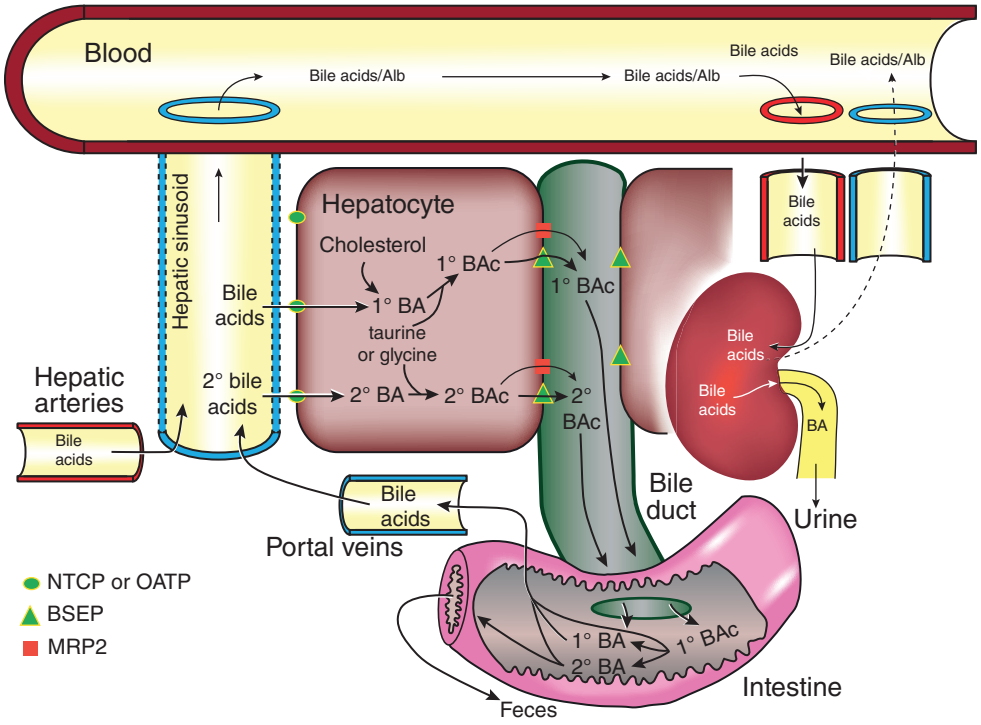


Figure 13.3. Physiologic processes of BAs. Cholesterol is degraded in hepatocytes to a 1° BA, either cholic or chenodeoxycholic acid. The 1° BA then becomes conjugated, usually with taurine or glycine, but sometimes with sulfate or gluconate, to form a 1° BAc. A 1° BAc is secreted into the biliary system, mostly by the ATP-coupled bile salt export pump (BSEP), and transported in micelles formed with cholesterol and phospholipid via the bile ducts to the intestine. In the intestine, a 1° BAc has three possible fates: (i) absorption by the intestinal mucosa and entrance into the portal blood, (ii) deconjugation by enteric bacteria to a 1° BA and then absorption by the intestinal mucosa and entrance into the portal blood, or (iii) degradation to a 2° BA (either deoxycholic acid or lithocholic acid), which may have two fates – excretion in the feces (minor) or absorption by apical Na-dependent BA transporters (ASBTs), most expressed in the ileal mucosa (not shown), with subsequent entrance into portal blood via basolateral organic solute transporters (OSTs) (not shown). If conjugated with sulfate, BAs are poorly absorbed by the intestinal mucosa. When returned to the liver via the portal blood, conjugated BAs, deconjugated BAs, and 2° BAs are efficiently removed from the blood, mostly by hepatocyte sodium-taurocholate cotransporting polypeptide (NTCP) (conjugated BAs) but also by Na^+-independent organic anion transporting polypeptides (OATPs, for unconjugated BAs); OATPs are also the major uptake pathway for Bu. The deconjugated BAs and 2° BAs are conjugated by hepatocytes and returned to the biliary system by BSEPs and multidrug resistance-associated protein 2 (MRP2) to complete the enterohepatic circulation. In health, nearly all BA molecules are within the enterohepatic circulation; very few are in the systemic blood, though the plasma [BA] transiently increases postprandially. In plasma, most BA molecules are bound to albumin and other proteins, including lipoproteins, but the more H_2O-soluble BAs that escape enterohepatic circulation can pass through the glomerular filtration barrier and be excreted in the urine; if not cleared, they return to systemic circulation.

1° BA, primary bile acid; 1° BAc, conjugated primary bile acid; 2° BA, secondary bile acid; Bile acids/Alb, bile acids associated with albumin

C. Taurine is an essential amino acid in cats, and it is necessary to maintain typical BA production. The bile of cats with dietary taurine deficiency contained markedly reduced total [BAs] and percentage of taurine-conjugated BAs.[38]

D. Bile salt secretion from hepatocytes to canaliculi occurs through two processes, one Na^+ dependent and one not, and is the major osmotic driving force for movement of H_2O and solutes into bile canaliculi (i.e., for bile production).

E. In health, the enterohepatic circulation of BAs is highly efficient, and nearly all bile salts excreted in bile are returned to the liver in one form or another via intestinal absorption and portal blood flow. Most of the BAs in blood are bound to albumin and other proteins, including lipoproteins, thus limiting renal excretion.[39,40] However, decreased hepatobiliary clearance of BAs can increase renal BA excretion and the ratio of urine [BA] to urine [creatinine].

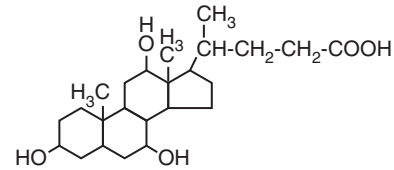
F. Bile salts are the major solids in bile that enter the intestine after gallbladder contraction. After intestinal reabsorption, the resulting higher [BAs] in portal blood may exceed the liver's extraction ability, thus yielding a postprandial increase in [BAs] in systemic blood. Typically, gallbladder contraction results from actions of cholecystokinin that is released after ingestion of a meal. However, gallbladder contraction may occur at other times.

G. The [BAs] is greater in foals than in adult horses and decreases in parallel with [Bt] and [Bu] over the first 3 wk of life.[41]

II. Analytical concepts

A. Unit conversion[8]

1. Total BAs: mg/L × 2.547 = µmol/L (SI unit)
2. Cholic acid: mg/L × 2.448 = µmol/L (SI unit)
3. Chenodeoxycholic acid: mg/L × 2.547 = µmol/L (SI unit)
4. Deoxycholic acid: mg/L × 2.547 = µmol/L (SI unit)
5. Lithocholic acid: mg/L × 2.656 = µmol/L (SI unit)
6. Occasionally, other units are used (i.e., mg/mL and mmol/L). See Table 1.6 for the conversion factors.



Cholic acid

B. Assays

1. Serum or plasma [BAs] is measured in clinical laboratories by spectrophotometric enzymatic assays that use 3α-hydroxysteroid dehydrogenase in reactions with conjugated and unconjugated primary and secondary BA forms. Free Hgb in a hemolyzed sample causes negative interference. Lipids in a lipemic sample create unacceptable errors according to one manufacturer.
2. The concentration of conjugated BAs can be measured by either radioimmunoassays or enzyme immunoassays, assays that may be economical for large reference laboratories.
3. Clinically, abnormalities in total [BAs] and conjugated BA concentration tend to parallel each other, and thus either type of assay can be used.
4. An in-clinic IDEXX SNAP BA immunoassay with automated reader is available to screen animals for increased [BAs]. Results are provided as < 5 µmol/L, > 30 µmol/L, or as an integer in the 5–30 µmol/L range. In one study, approximately 1/6 to 1/5 of

samples with a [BAs] ≤ 20 µmol/L based on a reference method had results > 20 µmol/L (considered increased) with the SNAP test (i.e., apparent false positives). However, all samples with a [BAs] > 20 µmol/L with the reference method yielded SNAP results > 20 µmol/L as well (i.e., no false negatives). These results are consistent with its use as a screening test to be followed by a quantitative reference method when values appear increased.[42]

5. A point-of care competitive fluoroimmunoassay using v-BA cartridges and the FUJIFILM DRI-CHEM IMMUNO AU10V analyzer based on surface plasmon-enhanced fluorescence showed good agreement with a 3α-hydroxysteroid dehydrogenase method for canine and feline plasma [BAs].[43,44]
   a. Limitations of the assay: Lipemia and marked hyperbilirubinemia may cause false increases. The analytical range limits numeric results to < 150 µmol/L, and sample dilution cannot be used because of changes in viscosity. No practical control material is currently available.
   b. Assay principle: In this assay, fluorescent particles coated with monoclonal antibodies to the three major feline and canine BAs (taurocholic, taurodeoxycholic, and taurochenodeoxycholic acids) bind BAs in a sample prior to interacting with albumin-associated BAs on a gold film in the reaction chamber. The more BAs in the sample, the fewer open antibody binding sites remaining on the particles, and the fewer the fluorescent particles that will bind to the surface-bound BAs in the reaction chamber. After incubation, laser-irradiation of the gold film from an appropriate angle below the film generates near-surface light on the BA-coated side of the metal via surface plasmon resonance, and that causes bound fluorescent particles to emit light; free particles are not affected, so washing is not needed. The light detected above the metal film is inversely proportional to the [BAs] in the sample.
   c. (Note: A surface plasmon is a quantum of collective electron oscillations around positive ions at the surface of a metal. Metals are neutral on average, but local charge imbalances within them cause electrons to oscillate around positive charges, thus producing plasmon resonance.)
6. Although ursodeoxycholic acid (Ursodiol) used for treatment of hepatobiliary diseases is detectable by routine assays for 3α-hydroxylated BAs, any increases in fasting or postprandial [BAs] after short-term or long-term administration to healthy dogs have been minimal and clinically insignificant.[45] Less positive interference was detected with the FUJIFILM plasmon-enhanced fluorescence method.[43,44] It is possible that greater effects occur in animals with hepatobiliary disease.
7. BA testing is more common than other liver function tests, such as plasma [NH_4^+] or dye-excretion tests, because it is relatively simple, it does not require special sample collection or handling, and point-of-care testing has been available.
8. Concentrations of specific unconjugated and conjugated BAs (fractionated bilirubin) can be measured in serum, bile, cavitary fluids, and feces by chromatographic and mass spectrometric methods. However, such testing in veterinary medicine is generally restricted to research.

C. Stability: Limited data are available for the stability of serum [BAs] in samples from domestic mammals. Sera from healthy dogs yielded only minor changes when samples were stored at –20 °C or –70 °C for 8 mo[46] and when whole blood or serum were stored at 4 °C for 1 d

Table 13.8. Diseases and conditions that cause an increased fasting [BAs]
Decreased BA clearance from portal blood
↓ Functional hepatic mass:* diffuse hepatocellular disease
↓ Portal blood flow to liver:* congenital and acquired portosystemic shunts, hepatic microvascular dysplasia
Decreased BA excretion in bile
Obstructive cholestasis
Hepatic cholestasis:* lipidosis, diabetes mellitus, steroid hepatopathy, hepatic lymphoma or other neoplasms, histoplasmosis, cytauxzoonosis, liver flukes affecting liver bile ducts, cirrhosis, cholangitis, cholangiohepatitis, periportal hepatitis, pyrrolizidine alkaloid toxicosis, sporodesmin toxicosis, alsike clover (*Trifolium hybridum*) toxicosis
Posthepatic cholestasis:* cholangitis, bile duct carcinoma and other neoplasms or cysts, liver flukes or rarely helminths in common bile duct, cholelithiasis, cholecystitis, congenital hepatobiliary malformations, pancreatitis (acute inflammation or fibrosis), pancreatic carcinoma, intestinal foreign body or neoplasm
Functional cholestasis (sepsis-associated cholestasis)

* A relatively common disorder, condition, or mechanism

Notes: Lists of specific disorders or conditions are not complete but are provided to give examples. Serum or plasma [BAs] may be increased postprandially or if factors other than eating stimulate gallbladder contraction. The [BAs] in foals is greater than adult horses during the first few weeks of life.

followed by 20 °C for 2 d.[47] Similarly, when whole blood and serum samples from healthy horses were stored at 20–25 °C for 3 d, changes in [BAs] did not appear to be clinically significant.[48]

III. Increased bile acid (BA) concentration in serum or plasma (Table 13.8)

A. An increased [BAs] can be called *hypercholemia* or *hypercholanemia*, but the terms are not commonly used in veterinary medicine. These terms should not be confused with hyperchloremia or hypercholesterolemia.

B. There are two major pathologic processes that increase serum [BAs] in fasting dogs and cats: (i) decreased BA clearance from portal blood and (ii) decreased biliary excretion of BAs, which may be accompanied by increased export of hepatocyte BAs to sinusoidal blood (Figure 13.4). Accordingly, increased serum [BAs] supports the conclusion that an animal has decreased hepatobiliary function. Increased serum [BAs] has high diagnostic sensitivity for hepatobiliary dysfunction. However, many primary and secondary liver diseases can cause the dysfunction, the dysfunctional state may or may not be reversible, and the magnitude of increase in [BAs] overlaps considerably among disorders.[49–51]

1. Decreased BA clearance from portal blood

a. Many pathologic states (e.g., inflammatory, metabolic, and degenerative) involving hepatocytes cause sufficient damage to reduce functional hepatic mass enough to impair BA clearance.[49–54]

b. Congenital and acquired portosystemic shunts or microvascular dysplasia enable bile salts absorbed in the intestine to bypass the liver, escape the enterohepatic circulation, and enter the systemic blood.[55]

c. Mild increases in [BAs] may occur when feed is withheld from horses.[56] The increase is probably caused by a decreased hepatic clearance rate of BAs.[18]

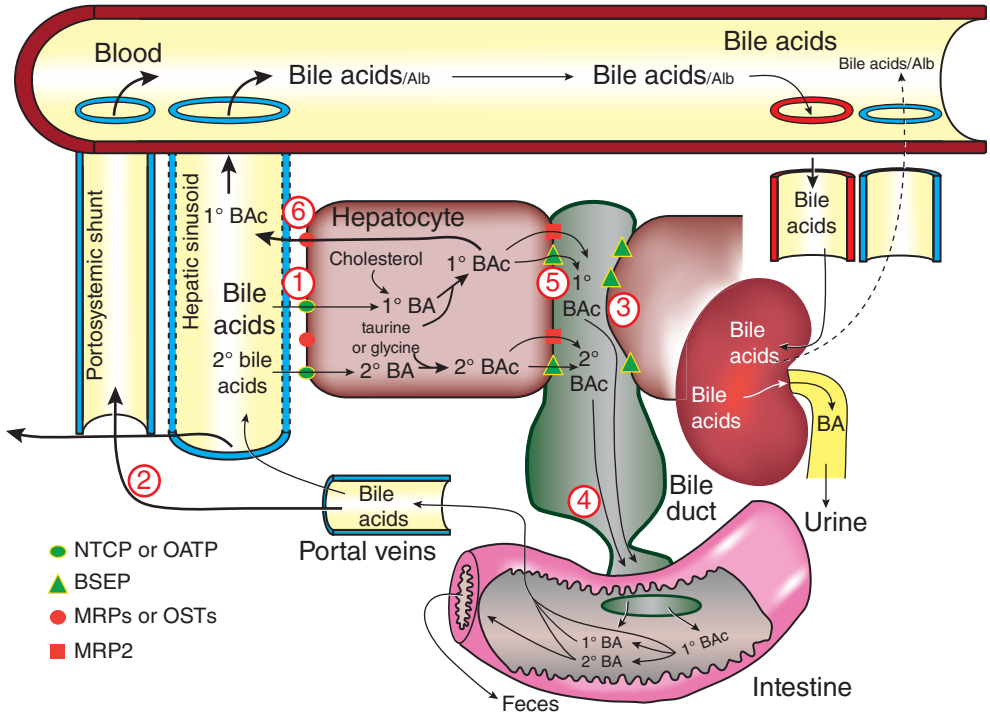


Figure 13.4. Pathologic processes that increase serum [BA] (hypercholemia). Two major pathologic processes cause hypercholemia:

- Decreased clearance from the portal blood: The defect may occur because of ① decreased uptake of BA from the sinusoidal blood because of a decreased functional hepatic mass or because ② there is a portosystemic shunt or hepatic microvascular dysplasia.
- Decreased biliary BA excretion: When there is ③ obstructive hepatic, ④ obstructive posthepatic, or ⑤ functional cholestasis, the BAs increase in sinusoidal blood instead of passing through the biliary ducts to the intestine. This occurs because of impaired bile flow, decreased BA secretion, and ⑥ BA-mediated upregulation of membrane transporters that can move BAs from hepatocytes to sinusoidal blood, thus decreasing hepatic toxicity and allowing for renal excretion. These transporters include organic solute transporters (OSTα, OSTβ) and certain multidrug resistance-associated proteins (MRP3 and MRP4). With cholestasis, hyperbilirubinemia would occur (not shown).

With hypercholemic disorders, more of the BAs become sulfated, making them more soluble and less bound to albumin or other proteins, thus facilitating their renal excretion.

BSEP, bile salt export pump; NTCP, sodium-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptides; Bile acids/Alb, bile acids associated with albumin

2. Decreased biliary BA excretion
   a. A variety of hepatic and posthepatic disorders can impair bile flow and thus impair excretion of BAs. Concurrent impairment of Bc excretion is expected, but it may not be enough to cause hyperbilirubinemia. Similarly, when hepatic or posthepatic hyperbilirubinemia is present, increased serum or plasma [BAs] is expected.
   b. During obstructive cholestasis, there is a down-regulation of canalicular bile salt transport proteins, and hepatocytes may pump bile salts into sinusoidal blood instead of into canaliculi.[32,57,58] This allows for renal excretion of BAs.

c. BAs are toxic to cells. Thus, accumulation of BAs in the liver may lead to cell damage and release of BAs and other substances from canaliculi and hepatocytes.
d. Cytokines (notably tumor necrosis factor α) have been shown to decrease bile salt transport proteins in hepatocyte canalicular membranes and thus impair BA secretion.[59,60] The resultant impairment in bile production is called *functional cholestasis* (see Bilirubin Concentration, Section III.G.3).

C. Mild increases in serum [BAs] occur in healthy animals after meals. Postprandial increases are exaggerated in some animals with hepatobiliary disorders. These findings are the basis of the BA challenge test (see Section IV).
D. In horses, increased serum [BAs] was found in nearly all horses (36 of 38) with hepatic necrosis, lipidosis, neoplasia, or cirrhosis. In contrast, only 2 of 78 sick horses without liver disease had increased serum [BAs] (RI not stated but upper limit estimated from a graph to be 18 μmol/L).[54] Hepatic BA clearance decreased by 36 % after ponies were fasted for 3 d, and the plasma [BAs] increased by 72 %, a much smaller increase than the increase in bilirubin concentration.[61]
E. In cattle, the diagnostic sensitivity of serum [BAs] varied among liver disorders: fascioliasis (100 %, n = 11), biliary calculi (100 %, n = 2), hepatic abscesses (53 %, n = 15), leptospirosis (71 %, n = 7), and hepatic lipidosis (86 %, n = 36) (RI not stated but upper limit estimated from a graph to be 40 μmol/L).[53] In another report, values for serum [BAs] were increased in cattle with hepatic lipidosis, hepatic abscesses, leptospirosis, and fascioliasis with mean concentrations of 90–225 μmol/L at initial evaluation.[62]
F. The diagnostic value of [BAs] in llamas and alpacas is largely unknown, but it is expected to be a marker of hepatobiliary disease. In five llamas that developed mild (two), moderate (two), or severe (one) hepatic lipidosis secondary to feed restriction and underlying metabolic demands, [BAs] was increased along with serum activities of AST, ID/SDH, and GGT, but [Bt] did not increase.

IV. Bile acid challenge tests (used in dogs and cats)
A. Principle: Stimulation of gallbladder contraction releases bile to the intestine and results in an influx of endogenous BAs to portal blood, thus challenging the liver to clear BAs and keep them in the enterohepatic circulation. Post-stimulation samples are generally paired with initial fasting (pre-challenge) samples. Post-challenge [BAs] may reveal impaired BA clearance that is not apparent or that is less clear with fasting samples.
B. Gallbladder contraction has typically been elicited physiologically by feeding a standardized small meal to induce cholecystokinin release. An alternative pharmacologic method has been reported in which the small meal is replaced by the peptide ceruletide, a cholecystokinin analog that stimulates smooth muscle and promotes gastric, biliary, and pancreatic secretion.[63] Use of ceruletide shortens the testing time (30-min post-ceruletide sampling), reduces variables such as those related to altered gastrointestinal function, and removes issues associated with giving food to anorectic or vomiting patients.
C. Procedure for postprandial BA challenge test
1. A fasting sample is collected from the dog or cat after a 12 h fast. Then, the animal is observed while it ingests food containing protein and fat (e.g., two teaspoons of a canned food for those < 10 lb, and two tablespoons for large dogs). A 2-h–postprandial sample is collected.
2. Sample hemolysis should be avoided during blood collection and processing because Hgb interferes with the spectrophotometric BA assay. Postprandial lipemia should be avoided by first collecting a fasting sample and then limiting the amount of ingested food as described.

D. Published data
1. Dogs[49]
a. The greatest fasting and postprandial [BAs] occurred in dogs with portosystemic shunts, cirrhosis, and cholestasis, although median values increased dramatically for portosystemic shunts and cirrhosis but not at all for cholestasis. Results overlapped considerably, so the samples did not consistently differentiate the type or severity of the disease. Some dogs with nonhepatobiliary disorders had an increased fasting or postprandial [BAs], but increases were minor (Table 13.9).
b. Fasting and postprandial samples both had high diagnostic sensitivity for portosystemic shunts (100 %) and moderate to high diagnostic sensitivity for hepatic disorders in general (not including shunts) (Table 13.10). For hepatic disease, the diagnostic sensitivity of fasting samples was slightly greater than that of

Table 13.9. Results of the BA challenge test in dogs with various disorders[49]

Disorder	N	[BAs], median (low-high)[a]	
		Fasting	Postprandial
Portosystemic shunt	29	114 (30 to > 250)	221 (48 to > 250)
Other hepatobiliary disorders	101		
Cholestasis	17	111 (0 to > 250)	111 (5 to > 250)
Cirrhosis	16	62 (0 to > 250)	149 (16 to > 250)
Hepatic necrosis	12	40 (4–160)	78 (51–188)
Chronic hepatitis	17	12 (0 to > 250)	74 (0 to > 250)
Steroid hepatopathy	9	12 (0–57)	19 (7–45)
Hepatic neoplasms	22	10 (0–179)	29 (0–199)
Passive congestion	8	5 (2–13)	5 (0–15)
Nonhepatobiliary disorders	40	3 (0–17)	6 (0–22)

[a] Units: µmol/L, reported here to the nearest 1 µmol/L
N, number

Table 13.10. Percentage of dogs and cats with increased BA challenge test results

Disorder	Dogs[a]			Cats[b]		
	N	% with ↑ [BAs]		N	% with ↑ [BAs]	
		Fasting	Postprandial		Fasting	Postprandial
Portosystemic shunt	29	100	100	24	79	100
Other hepatobiliary disorder	101	82.5	77.7	58	71	97
Nonhepatobiliary disorder	40	17.5	12.5	26	11.5	30.8

[a] Decision limit determined as the value 2 standard deviations above the mean for 66 healthy dogs: 5.0 µmol/L fasting and 15.5 µmol/L 2-h postprandial.[49]
[b] The method of establishing the decision limits was not reported, but they were 5.0 µmol/L for fasting samples and 10.0 µmol/L for postprandial samples.[50]
N, number

postprandial samples; however, the diagnostic specificity and positive predictive value were slightly greater for postprandial samples because of fewer increased postprandial results in nonhepatobiliary disorders.

c. When decision thresholds of 20 µmol/L for fasting samples and 25 µmol/L for postprandial samples were used, the diagnostic specificity of [BAs] for hepatobiliary disease including portosystemic shunts was 100 %, and the diagnostic sensitivities were about 60 % and 75 %, respectively (data from a total BA enzymatic assay; different decision thresholds would likely be needed for different assays).

d. The diagnostic utility of fasting and postprandial [BAs] may vary from the data provided here because of differences in (i) populations of dogs, (ii) BA assays, (iii) diagnostic decision limits (cutoffs), (iv) criteria for acceptable samples, and (v) diagnostic criteria for disorders.[64]

2. Cats[50]

a. The greatest median fasting and postprandial [BAs] results occurred in cats with cholestasis, portosystemic shunts, and hepatic lipidosis, and median [BAs] after food increased dramatically in these disorders. Results overlapped considerably, so the samples did not consistently differentiate the type or severity of the disease. Some cats with nonhepatobiliary disorders had an increased fasting or postprandial [BAs], but increases were minor (Table 13.11).

b. Postprandial samples had very high diagnostic sensitivity for portosystemic shunts and other hepatobiliary disorders when respective URLs were used, whereas fasting samples had moderately high diagnostic sensitivity for these conditions (Table 13.10).

c. The diagnostic specificity for the absence of liver disease approached 100 % when values 4 × URL and 2 × URL (i.e., 20 µmol/L for each) were considered abnormal for fasting or postprandial samples. However, with these decision limits, the diagnostic sensitivity for hepatobiliary disease was 49 % with fasting samples and 81 % with postprandial samples (data from a total BA enzymatic assay; different decision thresholds would likely be needed for different assays).

Table 13.11. Results of the BA challenge test in cats with various disorders[50]

Disorder	N	[BAs], median (low-high)[a]	
		Fasting	Postprandial
Portosystemic shunt	24	32 (0 to > 240)	140 (49 to > 250)
Other hepatobiliary disorders	58		
Cholestasis	17	40 (3 to > 250)	142 (6 to > 250)
Hepatic lipidosis	20	28 (0 to > 250)	142 (10 to > 250)
Hepatic necrosis	13	15 (0–178)	29 (3–176)
Hepatic neoplasms	8	8 (0–250)	26 (9 to > 250)
Nonhepatobiliary disorders	26	3 (0–16)	5 (0–15)

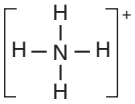
[a] Units: µmol/L, reported here to the nearest 1 µmol/L
N, number

E. Factors other than hepatobiliary and portal systems that influence results
1. Fasting [BAs]
a. Spontaneous contraction of the gallbladder might increase fasting [BAs] unexpectedly. However, in one small study, the sight and smell of food for 15 min did not increase [BAs] in canine serum samples collected 30–720 min later.[65]
b. Fasting serum [BAs] depends on the enterohepatic cycle, so it may be affected by intestinal disease, e.g., decreasing because of decreased intestinal absorption of BAs.
2. 2-h postprandial [BAs]
a. The peak [BAs] may not occur 2 h postprandially because of variations in gastric emptying, intestinal transit time, intestinal absorption, or other factors.
b. The animal may not have eaten the food provided, or it may have a poor cholecystokinin response.
c. The gallbladder may have contracted prior to feeding.
3. The aforementioned factors may contribute to the common finding that the fasting [BAs] is greater than the 2-h postprandial [BAs]. In such cases, if neither value is greater than the postprandial decision limit, there is no clear evidence of hepatobiliary disease. If either value is greater than the postprandial decision limit, there is evidence of hepatobiliary disease.

AMMONIUM (NH_4^+) CONCENTRATION IN PLASMA

I. Physiologic processes (Figure 13.5)

II. Analytical concepts
A. Units and terms
1. The analyte is commonly called "blood ammonia" (NH_3), but the dominant form in plasma is *ammonium* (NH_4^+), a cation. At a pH of 7.4 and a pK_a of 9.25, the NH_4^+:NH_3 ratio is about 70, and thus there is relatively little NH_3 in plasma. At body temperature and pressures, NH_3 is a gas.
2. Unit conversion[8]
a. NH_3: µg/dL × 0.5871 = µmol/L (SI unit)
b. NH_4^+: µg/dL × 0.5543 = µmol/L (SI unit)


Ammonium

B. Assays for NH_4^+ concentration
1. Methods
a. Enzymatic method: NH_4^+ + α-ketoglutarate + NADPH → glutamate + NADP via GMD, causing a decrease in absorbance
b. If an alkalinizing agent is used to convert NH_4^+ to NH_3, NH_3 can be measured by a dye-binding method. This method has been applied to point-of-care analyzers.
c. Other assays measure NH_4^+ via colorimetric or ion-selective electrode methods.
2. When [NH_4^+] values from two commercial analyzers were compared with values generated by the generally accepted enzymatic assay, there was good agreement between the Blood Ammonia Checker and the enzymatic assays but poor agreement between the VetTest and enzymatic assays.[66] A colorimetric point-of-care assay (PocketChem BA) had substantial proportional and systematic negative bias averaging –38 µmol/L when compared to a reference method, and the accuracy of its results for

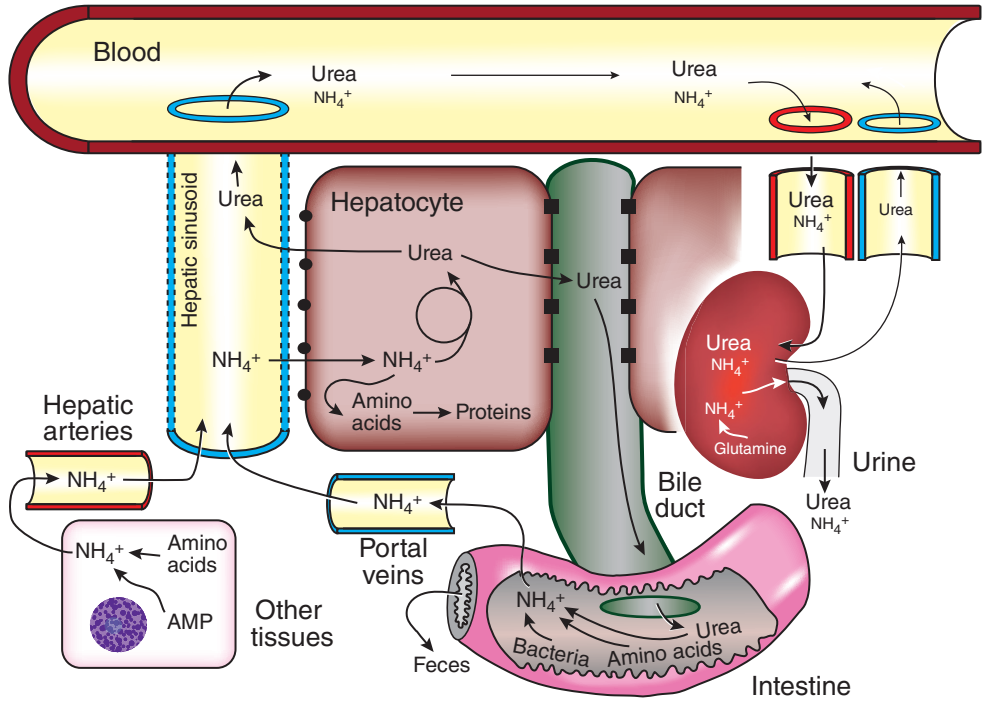


Figure 13.5. Physiologic processes of ammonium.

- Most NH_4^+ is produced in intestines by digestion of dietary proteins or by the metabolism of bacteria. Some NH_4^+ is produced by the deamination of amino acids (in many cells) and adenosine monophosphate (especially in myocytes).
- After NH_4^+ enters the liver (via the portal vein or the hepatic artery), it enters hepatocytes and is used for the synthesis of urea (in the urea cycle), amino acids, and proteins. Urea diffuses from hepatocytes to the sinusoidal blood or the bile canaliculi, from which it may be excreted via the kidneys or the intestine, respectively. Urea that enters the intestine (from diet, bile, or blood) may be reabsorbed as part of an enterohepatic circulation (also see Figure 8.6).
- Renal excretion of NH_4^+ may occur by NH_4^+ passing through the glomerular filtration barrier and being excreted in the urine. NH_4^+ is also fixed into glutamine in myocytes or other cells (not shown), and, in response to acidemia, deamination of glutamine in renal tubules results in NH_4^+ excretion (see Figure 9.7 for details).
- NH_4^+ is the molecular form that is present in most aqueous body fluids at a pH of 7.4, but it does not diffuse through cell membranes. NH_3 is relatively lipid soluble and rapidly diffuses across cell membranes, but very little is present in body fluids.

identifying hyperammonemia was 83.3 %, improving to 87 % when using a cutoff selected to correct for the bias.[67] However, whole blood was used for the point-of-care analysis while plasma was used for the reference method analysis. In a later study, plasma yielded greater values than whole blood when tested by a PocketChem BA, with about the same mean magnitude of difference (38 µmol/L), and plasma results were not significantly different from a reference method.[68] This emphasizes that RIs or other decision limits used for interpretation must be generated with the same sample type and sample handling of clinical samples, and appropriate RIs should be generated to interpret results of the PocketChem BA with each sample type of interest.

C. Samples
1. Routine clinical assays use plasma from EDTA- or heparin-anticoagulated blood samples, not whole blood or serum, but whole blood with or without anticoagulation has been used with point-of-care analysis.[68] The [NH_4^+] in serum samples is greater than in paired plasma samples.[69]
2. In healthy fasting dogs, the [NH_4^+] in arterial and venous plasma samples were not significantly different. However, in dogs with liver disease, the arterial samples had greater [NH_4^+] (see Ammonium Concentration, Section III.A.4).[70]
3. Hemolysis causes false increases of [NH_4^+] in some assays because the heme pigment interferes with light transmittance or because RBCs contain NH_4^+.[71] However, the effect of hemolysis might be substantially less than suggested by manufacturers if their information was based on studies using Hgb obtained by methods that promote NH_4^+ generation, e.g., osmotic lysis protocols with overnight storage allowing protein degradation.[72]
4. Sample handling
   a. Ideal: The laboratory is notified prior to blood collection to provide time to assess quality control solutions and assure that the assay will be ready for immediate use. EDTA- or heparin-anticoagulated blood is then delivered to the laboratory on ice, immediately after collection. The plasma is harvested by refrigerated centrifugation immediately after arrival, and chemical analysis is completed immediately (with storage at 4 °C if analysis must be delayed).[73]
   b. The plasma [NH_4^+] is usually considered to be stable for up to 4 h at 4 °C and adequately stable for 1–2 d at −20 °C.
   c. Suboptimal handling and instability problems can lead to highly variable results, typically increases over time.[74]
      (1) Within hours of storage at room temperature, the [NH_4^+] will be up to 2–3 times the starting value because of the generation of NH_4^+ from the degradation of labile proteins and amino acids (e.g., glutamine).
      (2) Delayed collection of plasma from the blood enables NH_4^+ to be produced by RBCs and leukocytes, thus increasing the measured [NH_4^+].
      (3) Delayed analysis of plasma enables $NH_{3(g)}$ to escape from plasma, particularly if the sample is not stoppered or if an evacuated tube is incompletely filled. Loss of $NH_{3(g)}$ will cause a decrease in the plasma [NH_4^+].

III. Hyperammonemia (Table 13.12)
A. Decreased clearance of NH_4^+ from portal blood
1. Disorders or conditions that reduce the removal of NH_4^+ from portal blood may cause hyperammonemia because intestinal bacteria produce large quantities of NH_4^+. Two major types of diseases have this pathologic defect:
   a. Diffuse hepatocellular diseases that reduce functional mass (e.g., cirrhosis, necrosis, and lipidosis)[51,52]
   b. Portosystemic shunts, either congenital or acquired[55,75,76]
   c. Increased [BAs] provides similar support for these conditions if cholestasis is not present; if cholestasis is present, [BAs] may be increased because of the cholestasis (decreased excretion), making [NH_4^+] the more useful test of hepatic function in that situation.
2. Decreased NH_4^+ clearance can be caused by congenital deficiencies that directly or indirectly involve the urea cycle (Figure 13.6). These disorders are rare.

Table 13.12. Disorders and conditions that cause hyperammonemia
Decreased NH_4^+ clearance from portal blood
↓ Functional mass:* diffuse hepatocellular disease
↓ Portal blood flow to liver:* congenital and acquired portosystemic shunts
Urea cycle enzyme deficiencies (congenital)
Cystinuria with concurrent arginuria and ornithinuria (reported in a cat)
Cobalamin deficiency
Increased NH_4^+ production
Intestinal disease in horses
L-Asparaginase treatment
Postprandial metabolism
Urea toxicosis in ruminants
Urea toxicosis in horses (rare)
Soybean meal toxicosis in ruminants (perhaps other protein meals too)
Strenuous exercise
Seizures
Increased NH_4^+ intake or absorption
NH_4Cl administration per os or per rectum
Ammoniated forage toxicosis in cattle
Exposure to NH_3 (gas) from anhydrous NH_3 (liquid)
Unknown or multifactorial mechanism
Urinary infection with urease-containing bacteria and concurrent urethral obstruction
Renal azotemia (documented in cats)

* A relatively common disorder, condition, or mechanism

Notes: Lists of specific disorders or conditions are not complete but are provided to give examples. A falsely increased [NH_4^+] may occur if delayed analysis of the sample results in proteolysis or NH_4^+ production by blood cells.

a. Argininosuccinate synthetase deficiency has been reported in dogs.[77] It has been suspected as a cause of transient subclinical hyperammonemia in Irish wolfhounds < 4 mo of age.[78,79] This finding can be misleading when screening such puppies for evidence of portosystemic shunting.
b. A probable case of ornithine carbamoyltransferase (ornithine transcarbamylase) deficiency caused hyperammonemia in a cat.[80]
c. Defective mitochondrial transport of ornithine, a substance that is transported from cytosol to mitochondria in the urea cycle, probably caused hyperammonemia, hyperornithinemia, and homocitrullinuria syndrome in Morgan fillies.[81]
d. Congenital cystinuria is caused by defective renal tubular resorption of cystine; concurrently, there is defective resorption of arginine and ornithine. A cystinuric cat had multiple episodes of hyperammonemic encephalopathy during its life.[82] The authors concluded that if dietary sources did not maintain sufficient amounts of arginine and ornithine for a functional urea cycle (Figure 13.6), then argininuria and ornithinuria caused a depletion of those amino acids, and the cat was then prone to hyperammonemia.

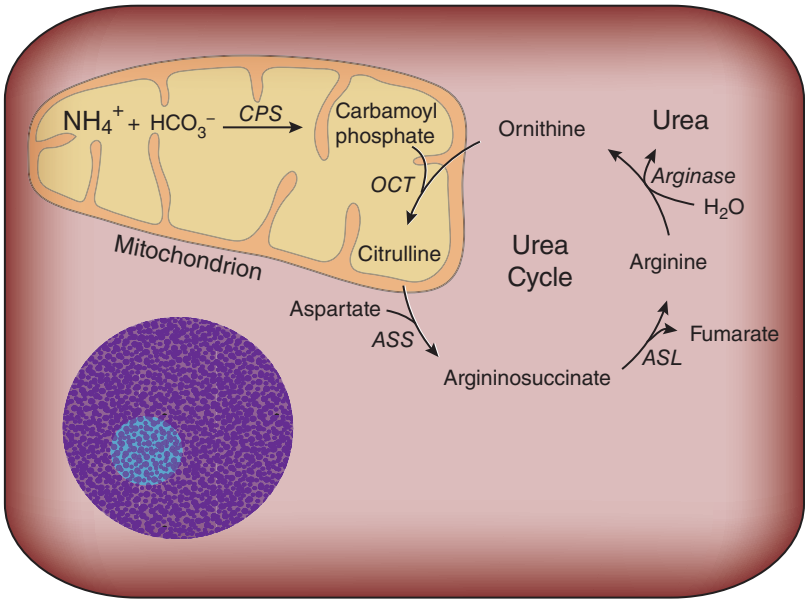


Figure 13.6. The urea cycle (Krebs–Henseleit cycle) in hepatocytes: Some reactions occur in mitochondria while others occur in a hepatocyte's cytoplasm. The roles of adenosine triphosphate (ATP) and phosphate are not shown.

- Carbamoyl phosphate synthetase (CPS) catalyzes the reaction in which NH_4^+ and HCO_3^- are used to produce carbamoyl phosphate. Ornithine carbamoyltransferase (OCT) catalyzes the combination of carbamoyl phosphate and ornithine to produce citrulline. Argininosuccinate synthetase (ASS) catalyzes the combination of citrulline with aspartate to form argininosuccinate (a link between the urea cycle and the citric acid cycle). Argininosuccinate lyase (ASL) catalyzes the generation of arginine and fumarate from argininosuccinate. Arginase catalyzes the combination of arginine and H_2O to urea and ornithine; ornithine can then enter a mitochondrion to continue the urea cycle.
- Congenital deficiencies in ASS or OCT, defective ornithine transport to mitochondria, and ornithine or arginine deficiency can each cause a defective urea cycle and hyperammonemia because NH_4^+ is not incorporated into urea.

3. Decreased NH_4^+ clearance can also occur with acquired disorders that cause a defective urea cycle (Figure 13.6). Cobalamin deficiency (see Chapter 15) promotes accumulation of methylmalonic acid, which decreases the concentrations of N-acetyl glutamate. A decreased N-acetyl glutamate concentration causes decreased activity of carbamoyl phosphate synthetase and thus less fixation of NH_4^+ into urea.[83]
4. In 69 dogs with hepatic encephalopathy caused by several liver diseases including 14 congenital and 29 acquired shunts, the $[NH_4^+]$ in arterial plasma averaged 1.5 times the $[NH_4^+]$ of venous plasma. The venous concentrations may be lower because of NH_4^+ clearance from blood perfusing kidneys, muscle, or other tissues. Because of the difference, some investigators recommend that arterial samples should be collected for measurement of plasma $[NH_4^+]$.[70] However, collecting an arterial sample is not as easy as collecting a venous sample. Also, based on the graphed data provided by the investigators, nearly all dogs that were hyperammonemic based on fasting arterial samples were also hyperammonemic based on fasting venous samples.

B. Increased NH_4^+ production
   1. Intestinal disease in horses: Horses (adults and foals) with intestinal disorders, most commonly colitis and/or enteritis, may have increased plasma [NH_4^+], sometimes marked, and clinical signs of colic and encephalopathy without evidence of hepatic disease.[84] An increased plasma [NH_4^+] is thought to be caused by increased generation of NH_4^+ by abnormal proliferations of colonic bacteria and/or by increased intestinal absorption of NH_4^+. No single type of bacterium has been cultured consistently in these cases. Diagnosis requires measurement of blood [NH_4^+]. Although a single URL has been used to identify hyperammonemia in adult horses and foals from multiple institutions,[84] appropriate RIs may vary considerably among laboratories, and it is not clear that adult reference values for [NH_4^+] also apply to foals; [NH_4^+] may be greater in healthy young children compared to older children and adults.[85]
   2. L-Asparaginase treatment: Hyperammonemia after L-asparaginase treatment of dogs and cats is thought to be caused by increased NH_4^+ production from the enzymatic degradation of L-asparagine, an amino acid, to aspartate and NH_4^+.[86] The hyperammonemia may last several days but is usually subclinical.
   3. Postprandial metabolism: Increased NH_4^+ production during the digestion of a meal may cause temporary hyperammonemia. This condition should not be considered if the animal is anorexic or was appropriately fasted prior to sample collection.
   4. Urea toxicosis in cattle: Urea toxicosis occurs with excessive ingestion of urea used as a nonprotein nitrogen feed source or fertilizer. In aqueous solutions, urea decomposition yields cyanate and NH_4^+. When the rumen pH is > 8.0, the NH_3:NH_4^+ ratio shifts toward NH_3, which diffuses from the rumen to plasma, where it combines with H^+ to increase the plasma [NH_4^+].[87] Hyperammonemia is not expected in young calves that have ingested urea prior to development of rumen function.
   5. Urea toxicosis in horses (rare): Adult ponies developed hyperammonemia after they were dosed with 450 g of urea; seven of eight ponies died.[88] Urea degradation to NH_4^+ presumably occurred in the cecum or colon by urease-producing bacteria, where urea may have reached because (i) it was not completely absorbed or degraded in the more proximal alimentary tract, or (ii) it was absorbed and then excreted into the distal intestinal tract.
   6. Soybean meal toxicosis in ruminants: Documentation of hyperammonemia after experimental soybean meal toxicosis in steers supports clinical findings of suspected toxicosis in ruminants ingesting large amounts of highly digestible protein in soybean or other protein meals.[89]
   7. Strenuous exercise: During exercise, two adenosine diphosphate (ADP) molecules combine to form adenosine monophosphate (AMP) and adenosine triphosphate (ATP) via the myokinase reaction of myocytes. The deamination of adenosine monophosphate produces inosine monophosphate and NH_3. The NH_3 diffuses to blood and increases the plasma [NH_4^+] and pH.[90]
      a. In greyhounds, the plasma [NH_4^+] increased from a mean of 82 μmol/L before a race to a mean of 256 μmol/L immediately after it.[91]
      b. In quarter horses, the plasma [NH_4^+] increased from a mean of 67 μmol/L before exercise (treadmill) to a mean of 137 μmol/L immediately after exercise.[92] In another study, the mean NH_4^+ concentrations before and after exercise were 37 and 113 μmol/L, respectively.[90] Hyperammonemia might have contributed to neurologic signs in two horses that became obtunded from endurance racing in severe heat.[93]

8. Seizures: Transient post-seizure hyperammonemia has been documented in cats lacking evidence for other causes of hyperammonemia.[94] As with strenuous exercise, muscle activity increases NH_3 generation.

C. Increased NH_4^+ intake or absorption
1. NH_4Cl administered per os or via the colon (see the next Section IV, NH_4^+ tolerance test)
2. Ammoniated forage toxicosis in calves: A 30-d-old calf had a plasma [NH_4^+] that was considered increased while nursing a cow that had ingested hay treated with anhydrous NH_3.[95]
3. Cattle dying after exposure to anhydrous NH_3 presumably have increased plasma [NH_4^+]. Cattle exposure occurs with the malicious release of anhydrous NH_3 from storage tanks.[96]

D. Unknown or multifactorial mechanism
1. Urinary infection with urease-containing bacteria and concurrent urethral obstruction: An azotemic dog with staphylococcal urinary tract infection and urethral calculi had a fasting [NH_4^+] of 258 μg/dL. The hyperammonemia may have been caused by increased NH_4^+ production in the urinary tract, decreased renal excretion due to obstruction, and perhaps decreased urea cycle activity due to acidosis.[97] Hyperammonemia has been reported in people with obstructive urinary tract infections not involving urease-producing bacteria.[98]
2. Renal azotemia: Hyperammonemia occurs in some cats with marked renal azotemia.[99] In 18 cats with renal azotemia, [NH_4^+] correlated with concentrations of urea nitrogen, creatinine, and phosphorus, but the specific cause of hyperammonemia remains to be determined.

IV. NH_4^+ tolerance test

A. Principle: By administration of NH_4Cl either orally or rectally, a challenge dose of NH_4^+ is presented to the liver via the portal veins. Either decreased hepatic functional mass or a portosystemic shunt will enable NH_4^+ to escape the enterohepatic circulation and cause an excessive increase in the plasma [NH_4^+].

B. The NH_4^+ tolerance test may be used when decreased hepatic function is suspected but other laboratory results do not strongly support hepatic insufficiency or a portosystemic shunt. It is particularly useful for excluding portosystemic shunts in dogs with suggestive symptoms.[76] Typically, an NH_4^+ tolerance test is not indicated if there is fasting hyperammonemia. Also, an excessively high plasma [NH_4^+] might contribute to hepatic encephalopathy.

C. Basics of procedures in dogs
1. *Oral NH_4^+ tolerance test*: After a 12-h fast and collection of a fasting sample, NH_4Cl is administered orally (0.1 g/kg but not more than 3 g) in 20–50 mL of H_2O. A 30 min post-NH_4Cl blood sample is collected.
2. *Rectal NH_4^+ tolerance test*: After a 12-h fast and collection of a fasting sample, NH_4Cl is administered (5 % solution, 2 mL/kg) via a catheter inserted 20–35 cm into the colon. Blood collection 20 and 40 min later has been recommended,[100] but collection at only 40 min may be sufficient.[76]

D. Interpretation of results
1. Fasting [NH_4^+]
   a. Within RI: There is no evidence of hepatic insufficiency or a portosystemic shunt. These conditions could be present, but the production of NH_4^+ is not enough to overwhelm the weakened system. There is no evidence of a rare congenital urea cycle enzyme deficiency.
   b. Hyperammonemia: See Table 13.12 for potential pathogeneses.

Table 13.13. Oral NH_4^+ tolerance test results for six dogs with portosystemic shunts[101]

	$[NH_4^+]$ (µg/dL)			
Assay 1[a]	10 Healthy dogs[b]	Dog 1[c]	Dog 2[d]	Dog 3[d]
Fasting	88 ± 36[c]	100	370	260
30 min	155 ± 71	1000	1400	850
Assay 2[a]	10 Healthy dogs[b]	Dog 4[e]	Dog 5[d]	Dog 6[d]
Fasting	56 ± 14	100	250	125
30 min	76 ± 30	550	900	650

[a] Two sets of data are reported because of the lack of analytical agreement between the two NH_4^+ assays (note the different values in the healthy dog groups).
[b] All data for healthy dogs are expressed as mean ± standard deviation. The data may not have had normal distributions.
[c] The fasting $[NH_4^+]$ was within RI, and thus hepatic function was sufficient to handle the NH_4^+ load during the fasting state. The marked hyperammonemia in the 30-min sample supports a portosystemic shunt or hepatic insufficiency.
[d] Fasting hyperammonemia revealed the defective NH_4^+ clearance. The marked hyperammonemia in the 30-min sample would be expected because of influx of NH_4^+ into the system.
[e] The fasting $[NH_4^+]$ was mildly increased and thus not strong evidence of hepatic insufficiency or a portosystemic shunt. However, the $[NH_4^+]$ in the 30 min sample was more substantially increased and thus supportive of hepatic insufficiency or a portosystemic shunt.

2. Postchallenge $[NH_4^+]$
   a. Within RI: There is no evidence of hepatic insufficiency, portosystemic shunt, or a rare congenital urea cycle enzyme deficiency.
   b. Increased above RI: There may be reduced functional hepatic mass, portosystemic shunting, or both. Rarely a congenital or acquired urea cycle enzyme deficiency is present.

E. Example oral NH_4^+ tolerance test results for dogs with portosystemic shunts are listed in Table 13.13.

V. Postprandial NH_4^+ tolerance test for dogs[102]

A. This procedure is similar to the NH_4^+ tolerance test (see the preceding Section IV) except digested food provides the NH_4^+ for the tolerance testing. The major advantage over the NH_4^+ tolerance test is that the administration of NH_4Cl solution is considered stressful, and some dogs may vomit the solution if it is administered orally.

B. Basics of the procedure
   1. Withhold food for 24 h and then feed a commercial diet containing about 30 % protein to provide 33 kcal/kg (~25 % of the estimated daily metabolizable energy requirement). This estimate is adjusted for growing dogs.
   2. Blood is collected prior to feeding and at 2 h intervals (2, 4, 6, and 8 h) after feeding. Blood is chilled during processing, and the plasma $[NH_4^+]$ is measured immediately after plasma is harvested.

C. In the published study,[102] the postprandial $[NH_4^+]$ did not exceed 16 µmol/L in samples from healthy dogs. Dogs with hepatocellular disease frequently had a preprandial $[NH_4^+]$ and a postprandial $[NH_4^+]$ above 16 µmol/L (note different units from Table 13.13 for the NH_4^+ tolerance test) without significant change between preprandial and postprandial values; the maximum concentrations occurred 6 h postprandially. Dogs with portosystemic

shunts had preprandial ammonium concentrations up to 250 μmol/L (90th percentile near 150 μmol/L) and postprandial ammonium concentrations up to 400 μmol/L (90th percentile near 250 μmol/L) in the 2, 4, or 6 h samples.

D. The data support the use of this tolerance test to detect portosystemic shunts and that a 6-h sampling may be beneficial. This postprandial tolerance test had poor diagnostic sensitivity for hepatocellular disease. Appropriate laboratory-specific decision thresholds should be used for diagnostic investigations.

DYE-EXCRETION TESTS

Prior to availability of spectrophotometric assays for BAs, hepatic function was clinically assessed by excretion tests involving two organic anionic dyes: BSP (bromosulfophthalein or sulfobromophthalein) and ICG (indocyanine green). Excretion of these dyes depends on hepatic blood flow, hepatocyte uptake, conjugation (BSP but not ICG), excretion to the biliary system, and enterohepatic circulation (BSP but not ICG). BSP is transported into hepatocytes by organic anion-transporting polypeptides (OATPs), conjugated with glutathione, and excreted into bile by multidrug resistance-associated protein 2 (MRP2). ICG enters hepatocytes via OATPs and sodium–taurocholate cotransporter polypeptide (NTCP); it is excreted into bile by multidrug resistance-associated proteins (MDRs). Impaired excretion (caused by a defect in any step of the pathway) was detected as either an increased percent retention of the dye or an increased plasma half-life of the dye. The use of dye-excretion tests is limited in clinical diagnostic testing because BSP is no longer commercially available, ICG is expensive, and assessment of [BAs] and [Bt] provides essentially the same information about hepatic function.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 14

GLUCOSE, KETOAMINES, AND RELATED REGULATORY HORMONES (INSULIN, GLUCAGON, GROWTH HORMONE, AND INSULIN-LIKE GROWTH FACTORS-1 AND 2)

Michael A. Scott and Steven L. Stockham

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Table 14.1. Abbreviations and symbols in this chapter	
[x]	x concentration (x = analyte)
ACTH	Adrenocorticotropic hormone (corticotropin)
DM	Diabetes mellitus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids (free fatty acids, nonesterified fatty acids)
GH	Growth hormone (somatotropin)
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter (facilitative hexose transporter)
Hct	Hematocrit (used synonymously with packed cell volume)
Hgb	Hemoglobin
IGF-1	Insulin-like growth factor-1 (somatomedin C)
IGF-2	Insulin-like growth factor-2
IGFBP	Insulin-like growth factor binding protein
IRG	Immunoreactive glucagon
IRI	Immunoreactive insulin
IRI:G	Immunoreactive insulin to glucose
LPL	Lipoprotein lipase
NaF	Sodium fluoride
PPID	Pituitary pars intermedia dysfunction
RBC	Red blood cell (erythrocyte)
RI	Reference interval
SI	Système international d'unités (International System of Units)
WBC	White blood cell (leukocyte)

GLUCOSE CONCENTRATION IN SERUM, PLASMA, OR WHOLE BLOOD

I. Physiologic processes (Figure 14.1)
   A. Blood [glucose] is regulated and influenced by several hormones. For the hormones to influence metabolism, there must be appropriate receptors and transport systems in the target cells. The following hormones have clinical relevance in veterinary medicine; there are others that also have roles in carbohydrate metabolism.[2]
      1. Insulin activity lowers blood [glucose] by promoting the uptake, utilization, or storage of glucose by hepatocytes, myocytes, and adipocytes. Insulin is not needed for glucose transport into neurons, WBCs, RBCs, platelets, or hepatocytes. Insulin does influence hepatocyte glucose uptake by altering activities of hepatic enzymes that promote glycolysis or glycogen synthesis, or by reducing gluconeogenesis.
      2. IGF-1 (somatomedin C) binds to its receptor on most cell types (hepatocytes expressing few receptors) and promotes cellular uptake of glucose. It indirectly promotes insulin activity in hepatocytes by inhibiting GH synthesis and secretion.
      3. IGF-2 can bind to insulin receptors and IGF-1 receptors and thus initiate corresponding actions on glucose. This is particularly important during prenatal development.

4. Glucose entry into most cells is modulated by a family of proteins called facilitative hexose transporters (GLUTs, for glucose transporters). Insulin promotes glucose entry into skeletal and cardiac myocytes and adipocytes via GLUT4; insulin is not needed for the other carriers (e.g., GLUT2 in hepatocytes). Human RBCs, RBCs from other species that cannot synthesize ascorbic acid from glucose, and RBCs of some adult dogs are known to express GLUT1. However, RBCs of most mammals do not appear to express GLUT1 after the neonatal period.[3] Instead, RBCs of at least some of these mammals express GLUT4, e.g., some Akitas; GLUT4 was not detected in RBCs of 6-mo-old calves.[4] Evidence of insulin effects on glucose uptake by RBCs of domestic species was not found.
5. Glucagon activity increases blood [glucose] by stimulating gluconeogenesis and glycogenolysis.
6. Catecholamine activity alters blood [glucose] by several mechanisms.[5]
   a. A β-adrenergic stimulus of pancreatic β cells increases insulin release. A β_2-adrenergic stimulus also increases hepatocyte glycogenolysis and gluconeogenesis.[6] After an immediate release of glucose by glycogenolysis, epinephrine mediates a sustained increase in gluconeogenesis, largely via lactate metabolism in the liver and kidneys.[7]
   b. An α_2-adrenergic stimulus of pancreatic β cells decreases insulin release and thus reduces glucose utilization by hepatocytes, myocytes, and adipocytes.[8] Because α_2-adrenergic receptors predominate on pancreatic β cells, this effect is greater than the β-adrenergic stimulus of pancreatic β cells to increase insulin release.
   c. An α_2-adrenergic stimulus of the pituitary gland increases the release of GH-releasing hormone, which then causes GH release.[9]
   d. An α_1-adrenergic stimulus promotes ACTH secretion and therefore hypercortisolemia and associated hyperglycemic effects including glycogenolysis and gluconeogenesis.[8,10,11]
7. GH (somatotropin) activity increases blood [glucose] by reducing glucose uptake by myocytes and adipocytes. Somatostatin (GH-inhibiting hormone) decreases GH production and release.
8. Cortisol activity increases blood [glucose] by stimulating gluconeogenesis and creating a state of insulin resistance (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section III.B.1.c).

B. In mammals with a single-chambered stomach, gluconeogenesis maintains fasting blood [glucose] by using products of protein and lipid catabolism. In cats, as obligate carnivores adapted to low-carbohydrate diets and periods of fasting, hepatic gluconeogenesis appears to be more continuously active and less regulated (they have no hepatic glucokinase) than in noncarnivores. These adaptations may promote insulin resistance and "stress-induced" hyperglycemia in cats.[1] In ruminants, propionate from rumen fermentation is used for hepatic gluconeogenesis. In horses, colonic propionate contributes to gluconeogenesis.[12]

C. Fasting normoglycemia represents a balance between the actions of insulin (promoting storage and utilization of fuels) and glucagon (promoting mobilization of fuels), with influences from other mediators, including cortisol, catecholamines, and GH. When there is an imbalance and fuel mobilization dominates, the animal will be hyperglycemic and potentially glucosuric; glucosuria reduces the magnitude of the hyperglycemia. If fuel storage and utilization are dominant, the animal will be hypoglycemic. Even though the balance of insulin and glucagon actions is assessed by a blood [glucose], these hormones also influence lipid and protein metabolism.

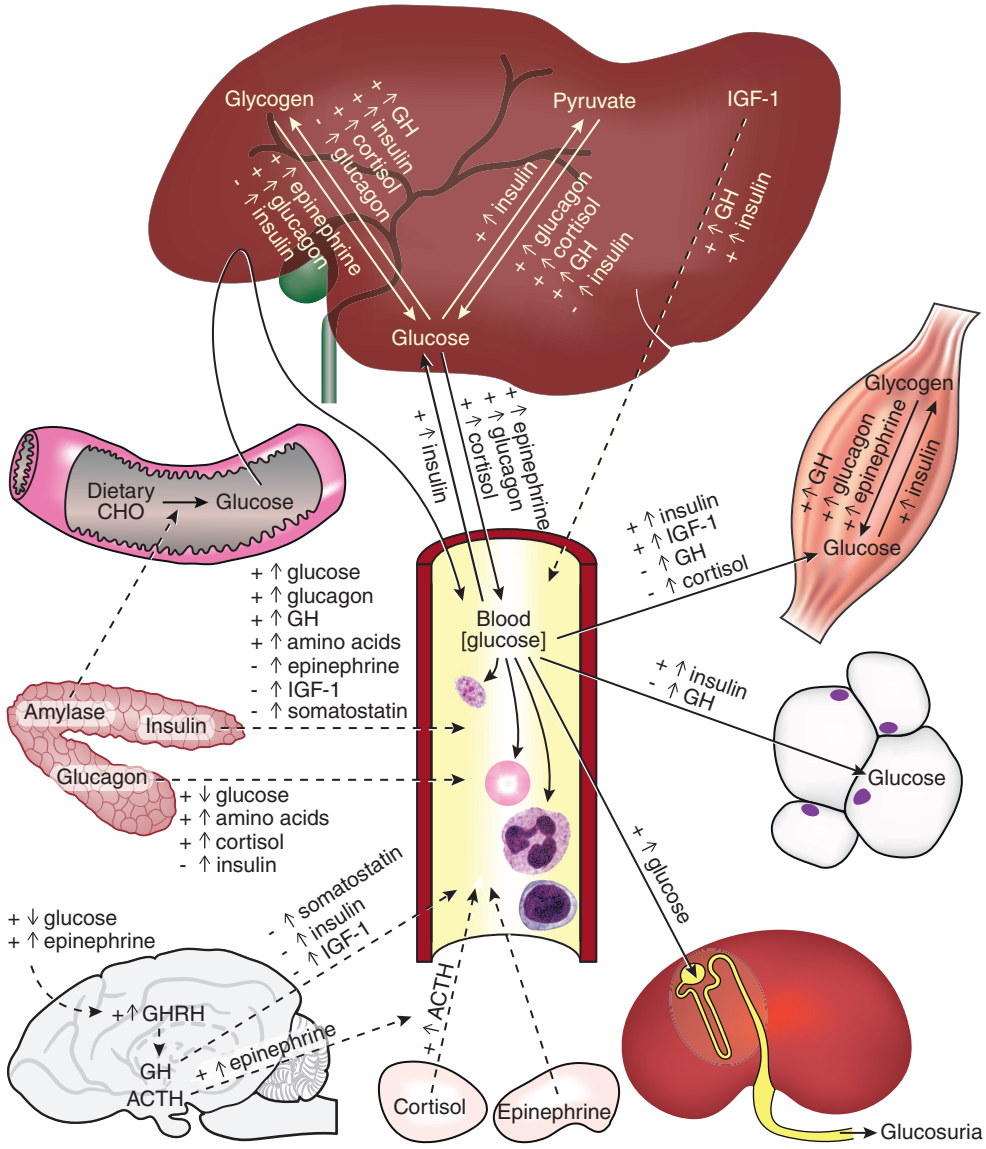


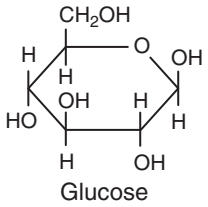
Figure 14.1. Physiologic factors that influence blood [glucose]. The major hormones affecting blood [glucose] are insulin (promotes glucose use and storage) and glucagon (promotes glucose production and release from storage). Several other hormones and other factors either directly or indirectly alter blood [glucose].

- Intestine: Dietary carbohydrates (CHO) are broken down to monosaccharides (including glucose) that are absorbed in the small intestine, from which they enter portal blood and then systemic blood if not removed by hepatocytes. Pancreatic α-amylase is released from the pancreas and catalyzes the breakdown of ingested starches to form glucose and other sugars.
- Pancreas: Insulin and glucagon are released from pancreatic islet β cells and α cells, respectively. Insulin secretion is primarily stimulated by increased blood [glucose], but its secretion is promoted by increased concentrations of other hormones and nutrients including glucagon, GH, and amino acids; secretion is inhibited by epinephrine, IGF-1, and somatostatin. Glucagon secretion is stimulated by hypoglycemia, increased amino acids, or increased cortisol; secretion is inhibited by increased insulin.

II. Analytical concepts

A. Terms and units

1. As explained in the following Section C, the [glucose] in whole blood may not be equal to its concentration in plasma or serum harvested from the same blood. Assuming similar contact times with blood cells, [glucose] in serum and plasma will be nearly equal to each other.
2. Unit conversion: mg/dL × 0.05551 = mmol/L (SI unit)[13]



B. Sample for [glucose]

1. Serum or plasma (especially heparinized) can be used. Serum and plasma should be removed from cells within 1 h of blood collection because glycolysis continues in blood cells in vitro, thus lowering [glucose]. Compared to other domestic mammals and people, equine blood has a longer clotting time and poorer clot retraction and thus more glucose may be consumed while waiting a sufficient time before removing serum from cells.[14]

Figure 14.1. *continued*

- Liver: Hepatocytes are the primary source of blood glucose during fasting. Glucose can be obtained from glycogenolysis (stimulated by epinephrine and glucagon; inhibited by insulin) or gluconeogenesis (stimulated by glucagon, cortisol, and GH; inhibited by insulin). Insulin also promotes glycolysis. Increased glucose release from hepatocytes is promoted by increased glucagon, cortisol, or epinephrine. Insulin promotes the hepatic uptake of glucose by promoting glucokinase activity (except in cats, which lack hepatic glucokinase),[1] but this glucose uptake does not require insulin. In cats, hepatic uptake of glucose is less responsive to increased [glucose] compared to noncarnivores, and gluconeogenesis is more continually active.[1] Insulin, cortisol, and GH promote glycogen synthesis. Insulin and GH stimulate the production of IGF-1 by hepatocytes.
- Muscle: Glucose uptake by myocytes is promoted by insulin through specific insulin receptors and GLUTs; IGF-1 also promotes glucose uptake but has much less activity. Increased GH and cortisol inhibit the uptake of glucose. Insulin promotes glycogen synthesis in myocytes, whereas GH, glucagon, and epinephrine promote glycogenolysis to provide glucose-6-phosphate for glycolysis. Myocytes do not contain glucose-6-phosphatase and thus glucose is not formed from glucose-6-phosphate and released to blood.
- Adipose tissue: Insulin promotes the uptake of glucose by adipocytes; GH reduces glucose uptake.
- Kidneys: In health, Na^+-glucose cotransporters in the proximal renal tubules resorb glucose, which passes freely through the glomerular filtration barrier. If the renal threshold for tubular resorption of glucose is exceeded, e.g., with physiologic hyperglycemia mediated by epinephrine and cortisol, then hyperglycemic glucosuria occurs.
- Brain: GH release from the pituitary gland is stimulated by growth hormone–releasing hormone (GHRH, somatocrinin), which is released from the hypothalamus during hypoglycemia or after epinephrine stimulation. Somatostatin, insulin, and IGF-1 reduce GH release.
- Adrenal glands: ACTH released in response to stress stimulates the adrenal cortex to release cortisol. The adrenal medulla releases epinephrine and norepinephrine in the fight-or-flight response. Epinephrine stimulates the release of ACTH from the pituitary gland.
- Blood cells: Glucose enters RBCs (not in pigs), WBCs, and platelets through insulin-independent processes and is used in glycolysis and the hexose monophosphate shunt.

Not shown: IGF-2 can bind to insulin receptors and IGF-1 receptors, thus initiating the responses associated with those hormones. Somatostatin is secreted by delta cells of pancreatic islets, duodenum, and stomach, as well as by certain cells in the brain. Progesterone stimulates the release of GH to plasma from canine mammary epithelial cells.

Note: The *solid arrows* indicate the movement of glucose; the *dashed arrows* represent the movement of hormones or α-amylase.

a. If plasma or serum has contact with cells at room temperature prior to centrifugation of blood, [glucose] typically decreases about 5–10 % per hour; marked leukocytosis and erythrocytosis accelerate the process. Although the rate of glucose consumption would be decreased by placing the blood sample in a cool environment, this is not recommended for clot tubes because clot formation and contraction would also be reduced. However, immediately chilling blood collected into a heparin tube is appropriate.
b. Serum separator tubes (tubes with gold, red, or red/black tops) contain an activator (e.g., siliceous substances) that enhances clot formation, and gel that enables easier separation of a blood clot and serum. After centrifugation, the serum [glucose] remains stable in a refrigerator for at least 48 h if the gel barrier is intact.[15,16] The gel barrier may break down in transit.
c. Collection tubes containing anticoagulants (oxalate or EDTA) and NaF (tubes with grey stoppers) have been used to inhibit glycolysis and reduce glucose consumption, but they are not effective enough to be recommended.[17] Promptly processed serum or heparin-plasma samples yield more accurate results.[18]
   (1) Fluoride inhibits glycolysis by complexing with Mg^{2+}, which is a cofactor for phosphopyruvate hydratase (synonym: enolase), an enzyme late in the glycolytic pathway. However, glucose is still metabolized by upstream glycolytic enzymes. Also, the more-inhibitory (but incompletely inhibitory) higher concentrations of NaF must be avoided because they induce more hemolysis.[19]
   (2) Plasma [glucose] decreases by about 5–15 % during the first hour after blood collection into NaF tubes, and decreases continue at a slower rate for another 3 h.[19,20] Besides ongoing glucose metabolism, RBC lysis and osmotic movement of H_2O from RBCs to plasma when the blood is mixed with the hyperosmotic salts (NaF and potassium oxalate) may also contribute to the decreases, particularly when tubes are underfilled; the spun Hct of promptly analyzed feline blood samples was substantially lower for blood collected into NaF/oxalate tubes compared to EDTA tubes.[20] After 4 h, fluoride inhibition of glycolysis stabilizes [glucose] for at least 3 d.[21]
   (3) Fluoride can interfere with glucose measurement by glucose oxidase-peroxidase methods, but this is overcome by appropriate reaction pH and peroxidase concentration.[22] Fluoride also inhibits Na^+-K^+ ATPase, which may, along with release of RBC contents (e.g., Hgb, inorganic phosphates, and K^+), lead to erroneous results of some clinical chemistry assays.

2. Whole blood is typically used in point-of-care instruments, including portable blood glucose meters developed to monitor the control of diabetic states in people or domestic mammals. In addition to routine venous samples, samples may be collected from ear veins, carpal pads, or the oral mucosa. In a study that used a veterinary coulometric test strip calibrated to report plasma equivalent [glucose] from whole blood samples, the [glucose] in carpal pad and oral mucosal samples from healthy dogs was significantly lower than in samples from the saphenous vein; 10 of 24 samples from the carpal pad incorrectly indicated the presence of hypoglycemia.[23]
3. The [glucose] in capillary and arterial blood is greater than in venous blood because tissues consume glucose. The difference in normoglycemic and normoinsulinemic states is probably < 10 mg/dL.

C. Whole blood versus plasma [glucose]: When an assay uses whole blood for a sample, the result may represent (i) a whole blood [glucose] (the concentration in plasma and cells combined), (ii) a plasma [glucose] (sample cells are excluded from the analysis), or (iii) a calculated plasma [glucose] equivalent (the expected plasma concentration of glucose based on the concentration measured in whole blood). Glucose that became bound to Hgb to form glycated Hgb is not measured and does not affect results.
   1. Whole blood and plasma glucose concentrations differ because the concentration of glucose in RBCs is lower than in plasma. This is because glucose is in only the H_2O compartment of RBCs and plasma, which is about two-thirds to three-fourths of the RBC volume but about 93 % of the plasma volume. Also, glucose enters RBCs by facilitated diffusion down a concentration gradient, so the concentration in RBC H_2O is never greater than in plasma H_2O, and in fact appears to be lower in domestic mammals. Overall, then, the higher the Hct, the greater the difference between plasma and whole blood [glucose], with whole blood [glucose] being lower than plasma or serum [glucose].
   2. Most instruments that measure a whole blood [glucose] calculate and report a plasma [glucose] equivalent to facilitate comparison with serum chemistry values (though many methods lack agreement). Some calculations assume a Hct that reflects health, so the calculated value is not correct in samples from anemic or erythrocytotic patients.[24] With certain analyzers, errors can be very clinically significant, falsely low with erythrocytosis and falsely high with anemia or when serum or plasma is used instead of whole blood.[25,26]
   3. Human whole blood [glucose] can be converted to plasma [glucose] by using a formula (Eq. 14.1) that factors in the Hct and the lower RBC [glucose] compared to plasma [glucose].[24] Different formulae may be appropriate for different species, but this makes the point that glucose results generated by certain analyzers should be interpreted cautiously when a patient's Hct is substantially increased or decreased.

$$\text{Plasma [glucose]} = \frac{\text{Whole blood [glucose]}}{1.0 - (0.0024 \times \text{Hct in \%})} \qquad (\mathbf{14.1})$$

   4. The differences that may occur between human whole blood [glucose] and plasma or serum [glucose] are listed in Table 14.2. When severe anemia is present, whole blood [glucose] and plasma [glucose] are nearly equal.
   5. Some whole blood assays measure molality and not molarity, and thus variations in the H_2O content of blood (e.g., due to displacement of H_2O by proteins or lipids) influence measured [glucose].[27,28]

D. Methods of measuring [glucose]
   1. Photometric assays: Most current methods use glucose oxidase (specific enzyme for glucose), glucose dehydrogenase, or hexokinase (enzyme for all hexoses) for the initial reaction. In other assays, glucose (and other monosaccharides) reacts with o-toluidine, or glucose reduces reagents such as copper or ferricyanide. Color changes can be detected by spectrophotometry or reflectance photometry.
      a. Lipemia, hemolysis, and icterus can interfere with the light transmission, but the degree of interference varies among assays.
      b. Rarely, IgM paraproteinemia has caused marked pseudohypoglycemia with use of hexokinase methods but not with electrochemical or dry-slide methods that avoid interference from protein precipitation. In people with IgM paraproteinemia, it was heparin that induced protein precipitation in two plasma samples, but similar false decreases have occurred with serum samples in people and a dog.[29]

Table 14.2. Calculated plasma [glucose] in a human blood sample with given whole blood concentration and Hct

	If whole blood [glucose] (mg/dL) is:				
	50	100	400	800	Increase above whole blood [glucose] (%)
If Hct is:	Calculated plasma [glucose] (mg/dL) is:				
10 %	51	102	410	820	2
45 %	56	112	448	897	12
60 %	58	117	467	935	17

Note: Concentrations of plasma glucose were calculated by using Eq. 14.1 and assuming that variations in sample handling and in vitro glycolysis were not present. Agreement between these calculated values and measured plasma concentrations would depend on other analytical properties of the assays (accuracy, precision, specificity, and analytical range). Conversion using Eq. 14.1 is applicable when the glucose assay result is a whole blood [glucose] in mg/dL or mmol/L. Other conversion factors are needed if the glucose assay measures molal concentration (mmol/kg) instead of molar concentration (mmol/L).

c. Isopropyl alcohol can produce a positive interference with reagent pad methods (e.g., Dextrostix) when blood is contaminated with isopropyl alcohol during blood collection; the resulting [glucose] can be greatly increased (change from 100 mg/dL to nearly 300 mg/dL).[30]

2. Nonphotometric assays
   a. Nonphotometric glucose oxidase methods have an advantage over photometric assays in that there are fewer chemical and photometric interferences. In the glucose oxidase reaction, O_2 is consumed, and hydrogen peroxide is produced. Some clinical laboratory and point-of-care instruments measure the rate of O_2 consumption with oxygen electrodes, and others measure the rate of hydrogen peroxide generation with hydrogen peroxide electrodes.
      (1) Some of the glucose oxidase methods are inhibited by fluoride or bromide; false decreases in [glucose] can be marked in samples from patients receiving potassium bromide for neurologic disease.
      (2) Because these assays are dependent on ambient O_2 pressure, older instruments were not reliable at high altitudes.[31] More recent assessment using a hypobaric chamber to simulate conditions at 8000 ft (and typical airplane cabin pressures) found no error attributable to the glucose oxidase method.[32] However, overestimation of [glucose] may occur at high altitudes, lower temperature, and lower relative humidity.[31]
   b. Nonphotometric glucose dehydrogenase methods also have similar advantages over photometric assays and are being used in point-of-care instruments. The amperometric assays are not dependent on ambient O_2 pressure.
3. Portable blood glucose meters
   a. Glucometers vary substantially and should be validated for each species. Many can provide useful results if the unit is operating correctly, if the sample is collected properly, and if manufacturer's directions are followed precisely (including sample type). Considerations regarding preanalytical errors, analytical errors, postanalytical errors, and quality assurance for glucometers have been reviewed.[33]

b. Glucometer inaccuracies (constant or proportional biases) have been common, so different RIs may be needed and single medical decision limits for [glucose] cannot be applied universally. Note: Many studies comparing glucose methods report high correlations with reference methods, but correlations can be excellent when agreement is poor (see Chapter 1, Evaluating and Validating Laboratory Methods, V).

4. Continuous glucose monitoring:[34] This involves use of transcutaneous or subcutaneous electrochemical sensors to measure [glucose] in interstitial fluid. It is used mostly to facilitate management of diabetic dogs and cats. It has also been evaluated in horses.[35] Most systems use a glucose oxidase method that generates hydrogen peroxide and produces an electrical current that increases with increasing [glucose]. Although significant analytical biases from reference methods for serum [glucose] have been reported repeatedly, particularly in the hypoglycemic range, continuous monitoring can be very useful. Advantages compared to generating serial blood [glucose] curves include no repeated venipunctures or use of an indwelling catheter, better detection of periods of hypoglycemia or hyperglycemia, less animal stress, and less time for staff. Disadvantages include initial expense of the system, limited range of [glucose] measurements (e.g., 40–400 mg/dL, 20–600 mg/dL, 20–500 mg/dL), potentially limited range for wireless communication, technical problems, and a lag time for equilibration of interstitial and plasma glucose concentrations (generally about 5–12 min in dogs and cats, up to 60 min in horses).[36,37] This lag time must be considered whenever [glucose] may change rapidly, e.g., with physiologic responses in cats, soon after insulin administration, or in critically ill foals.[37–40] Dehydration, thin canine skin, and sensor placement may also promote inaccuracy.[34]

III. Hyperglycemia

A. As summarized in the preceding Physiologic Processes section, plasma [glucose] is influenced by many factors, so it should not be surprising that there are many causes of hyperglycemia. Many hyperglycemic disorders can be placed under the umbrella of DM, but a definition and single classification system for DM have been lacking in veterinary medicine, in part because of knowledge gaps. An etiologic classification system for canine and feline DM has been proposed by the European Society for Veterinary Endocrinology (see esve.org/alive/search.aspx).

1. The definitions, diagnostic criteria, and classifications of DM used in this chapter were described in a report from a committee organized by the American Diabetes Association.[41]

a. DM is a pathologic state caused by a group of metabolic disorders and characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. In people, there are criteria for staging preclinical type 1 DM based on presence of autoantibodies (stages 1 and 2) and evidence of a hyperglycemic disorder (stage 2). Stage 3 is characterized by fasting hyperglycemia and associated clinical signs (e.g., polyuria, glucosuria). In veterinary medicine, clinical DM is typically diagnosed when there is persistent hyperglycemia and glucosuria along with associated clinical signs, e.g., polyuria, polydipsia, and weight loss.[42] Criteria for diagnosing a prediabetic state in cats have been described.[43]

b. Diagnostic criteria for human DM are shown in Table 14.3 to reflect the principles behind the detection of DM.[41] To adapt these criteria to domestic mammals, veterinarians will need to establish appropriate decision values for [glucose] in each

Table 14.3. Criteria for the diagnosis of diabetes mellitus in people[41]

1. Fasting plasma [glucose] ≥ 126 mg/dL (7 mmol/L); fasting is defined as no caloric intake for at least 8 h.[a,b]
Or
2. 2-h plasma [glucose] ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test;[c] the test should be performed as described by the World Health Organization, using a glucose load containing an equivalent of 75 g anhydrous glucose dissolved in water.[a]
Or
3. Hgb A1c ≥ 6.5 % (48 mmol/mol); the test should be performed in a laboratory using a method that is … certified and … standardized…[a]
Or
4. In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma [glucose] ≥ 200 mg/dL (11.1 mmol/L)[d]

[a] In the absence of unequivocal hyperglycemia, results should be confirmed by repeat testing.
[b] The decision limit of 126 mg/dL (7.0 mmol/L) was based on several factors, including correlation with results of glucose tolerance tests and complications of persistent hyperglycemia (e.g., retinopathy or arterial disease). Until similar studies are done in domestic species, similar decision limits cannot be established.
[c] It is unlikely that veterinarians need such a criterion in clinical medicine.
[d] Evidence-based criteria have not been established in veterinary medicine, and assay variability confounds universal decision limits, but the ≥ 200 mg/dL criterion may be reasonable for horses and has been proposed for dogs, while ≥ 270 mg/dL (15.0 mmol/L) and ≥ 150 mg/dL (8.3 mmol/L) have been proposed for cats and cattle, respectively. Classic symptoms of hyperglycemia include polyuria, polydipsia, and unexplained weight loss.

animal species (see below and Table 14.3 footnote for suggested decision thresholds for dogs, cats, horses, and cattle). In a study involving 79 healthy senior cats, the decision threshold for further evaluation of glucose homeostasis was 189 mg/dL for samples collected immediately at initial examination but 116 mg/dL for samples collected after overnight hospitalization and fasting for 18–24 h.[45] The European Society of Veterinary Endocrinology has proposed criteria for the diagnosis of DM in dogs and cats:[46,47]

(1) Dogs
   (a) Fasting or nonfasting blood [glucose] ≥ 200 mg/dL (11.1 mmol/L) in a dog in hyperglycemic crisis or with clinical signs of hyperglycemia and no other plausible cause of hyperglycemia; if clinical signs are unclear, assess for persistent hyperglycemia and/or glucosuria, or
   (b) Fasting blood [glucose] < 200 mg/dL and > 126 mg/dL (7 mmol/L) for > 24 h with or without signs of hyperglycemia or hyperglycemic crisis; hyperketoaminemia with no other apparent cause may be used to indicate persistent hyperglycemia (fasting defined as no food for ≥ 8 h)

(2) Cats (complicated by magnitude of physiologic hyperglycemia)
   (a) Fasting or nonfasting blood [glucose] ≥ 270 mg/dL (15.0 mmol/L) in a cat in hyperglycemic crisis or with clinical signs of hyperglycemia, no other plausible cause of hyperglycemia, and either or both of the following: (i) hyperketoaminemia with no other apparent cause, (ii) glucosuria documented at least twice using naturally voided samples in a home environment ≥ 2 d after any stressful events, or

(b) Fasting or nonfasting blood [glucose] > 126 mg/dL and ≤ 270 mg/dL in a cat with at least two of the following: (i) hyperglycemic crisis or clinical signs of hyperglycemia with no other plausible cause, (ii) hyperketoaminemia with no other apparent cause, and (iii) glucosuria documented at least twice using naturally voided samples in a home environment ≥ 2 d after any stressful events

(c) Equivocal findings may warrant re-evaluation.

2. The human classification system for DM provides a mechanistic framework that is largely applicable to veterinary medicine; modifications may be needed as understanding of the pathogeneses of DM in domestic mammals increases.
   a. Portions of the human classification system have been used to classify canine and feline DM into two major groups: (i) insulin-deficiency diabetes (IDD), which includes those disorders that cause a progressive loss of β cells (e.g., type 1 DM and pancreatitis), and (ii) insulin-resistance diabetes (IRD), which includes those disorders in which there is insulin antagonism by hormones (e.g., type 2 DM and DM secondary to other endocrine disorders).[44,48] However, this chapter uses the human classification system.
   b. Despite general applicability, using the human classifications of type 1 and type 2 DM in veterinary medicine may be considered inappropriate because the exact pathogeneses of the diabetic states are frequently not established. For example, immune-mediated destruction of islet β cells is the major pathogenesis of type 1 DM in people but strong evidence of similar autoimmunity is currently lacking in most dogs with insulin-deficiency DM.[49]
   c. Avoidance of "insulin-dependent" and "non–insulin-dependent" DM classifications has been recommended because of misunderstandings generated by their use.[50]
3. For the pathologic hyperglycemic disorders, several other abnormal laboratory findings may be present and can be used to help characterize the severity or duration of the diabetic states.
   a. Glucosuria and associated polyuria (Chapter 8)
   b. Ketonemia and ketonuria (Chapters 9 and 8, respectively)
   c. Metabolic acidosis (Chapters 9 and 10)
   d. Lipemia (Chapter 16)
   e. Increased [ketoamine] or ketoamine percentage (see Ketoamines: Fructosamine and Glycated Hemoglobin, Section III)

B. Disorders and conditions (Table 14.4)
1. Physiologic hyperglycemia
   a. Postprandial hyperglycemia: Glucose absorbed after carbohydrate (starch, simple sugar) digestion increases glucose entry into blood. Also, amino acids absorbed after protein digestion stimulate the release of glucagon which then promotes hyperglycemia via gluconeogenesis. The postprandial [glucose] in a monogastric animal should return to fasting values within 4 h, more quickly and to a lower baseline [glucose] with some diets than others, depending on the type and amount of carbohydrate in the diet.[51,52]
   b. Excitement or fright: Catecholamines (epinephrine and norepinephrine) stimulate glycogenolysis, decrease glucose utilization, and may promote hypercortisolemia (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section I.A.6). The magnitude of the hyperglycemia varies among species and is relatively high in cats

Table 14.4. Disorders and conditions that cause hyperglycemia
Physiologic hyperglycemia: postprandial, excitement, fright, glucocorticoid-associated, diestrus*
Pathologic hyperglycemia[a]
Type 1 DM: β-cell destruction, usually leading to insulin-deficiency DM
Idiopathic DM (major form in dogs)*
Immune-mediated DM
Type 2 DM: ranging from predominantly insulin-resistance DM to predominately an insulin secretory defect with insulin resistance (major form in cats)*
Pancreatic DM:* pancreatitis, pancreatic carcinoma, partial pancreatectomy
Other endocrine DM:* hypersomatotropism (acromegaly), glucagonoma, hyperadrenocorticism, hyperpituitarism, hyperthyroidism, hypothyroidism, pheochromocytoma, bovine milk fever, canine aminoaciduric hypoaminoacidemic hepatopathy syndrome (includes canine hepatocutaneous syndrome)
Drug-induced DM: glucocorticoids, thyroid hormone, megestrol acetate, medroxyprogesterone acetate, proligestone
Infectious DM: bovine virus diarrhea, sepsis, endotoxemia
Hyperammonemia: horses and cattle
Genetic DM: keeshonds, possibly Samoyed dogs
Anti-insulin antibodies
Pharmacologic or toxicologic hyperglycemia (transient)
Oral or intravenous glucose,* glucocorticoids, megestrol acetate, glucagon, thyroxine, ethylene glycol, α_2-adrenoceptor agonists (e.g., xylazine, detomidine, amitraz), octreotide, propranolol, albuterol, morphine, progestins, possibly insulin (Somogyi effect)

* A relatively common disorder, condition, or mechanism

[a] In the ADA classification,[41] pathologic hyperglycemias other than Type 1 and Type 2 DM are called "Other specific types of DM." Those recognized in veterinary medicine are listed in this table after Type 2 DM.

Note: Because of substantial differences in results generated by different methods and instruments (see Glucose Concentration in Serum, Plasma, or Whole Blood, Sections II.C and D), applicable RIs should be used to identify hyperglycemia.

(peak values near 300 mg/dL).[53] The major contributor to feline "stress-induced" hyperglycemia may be the physical activity of struggling that increases gluconeogenesis because of increased [L-lactate] formed in muscle during aerobic glycolysis, but decreased [insulin] and increased [norepinephrine], [glucagon], and [cortisol] likely contribute to maintaining hyperglycemia.[53] Cats may be predisposed to such hyperglycemia because they lack hepatic glucokinase.[1] Excitement with or without struggling may contribute to transient hyperglycemia in many patients, particularly those that are critically ill.

c. Glucocorticoid-associated hyperglycemia: Glucocorticoids produced by "stressed" patients increase release of FAs to blood, stimulate glucagon release and gluconeogenesis, and create a state of insulin resistance, especially in skeletal muscle, in part by inhibiting translocation of GLUT4 to cell membranes.[54] Endogenous release of cortisol likely contributes to hyperglycemia in many patients, including those that are critically ill. As noted later, these mechanisms also occur in pathologic

and pharmacologic hyperglycemias. In addition, with prolonged excess glucocorticoids, glucocorticoid-induced apoptosis of pancreatic β cells may occur.[55] Endogenous cortisol may induce marked hyperglycemia, glucosuria, and hyperosmolar syndrome in llama and alpaca crias.[56]

d. Diestrus: Progesterone released from the corpus luteum promotes release of GH, which decreases uptake of glucose by myocytes and adipocytes.[57] In dogs, this GH is produced by ductal epithelial cells of mammary glands and not by the pituitary gland.[58] Progestins have also been shown to stimulate expression of GH mRNA in feline mammary tissue.[59]

2. Pathologic hyperglycemia: Using the ADA human classification, pathologic hyperglycemia includes type 1 DM, type 2 DM, and "other specific types of DM." The other types that are recognized in veterinary medicine are discussed in this section after type 2 DM. Accurate classification of diabetic states is difficult when an animal with clinical features of Type 1 DM has a concurrent disorder causing insulin resistance.[60]

a. Type 1 DM: β-cell destruction, usually leading to absolute insulin deficiency (insulin-deficiency DM); previously called insulin-dependent DM, type I DM, or juvenile-onset DM

(1) This form of DM is caused by specific destruction of pancreatic β cells. The destruction is immune mediated in people.

(2) Insulin-deficiency DM similar to human type 1 DM is the most common form of DM in dogs, but evidence of an immune-mediated pathogenesis is limited and inconsistent.[49,61] Investigators have shown that some diabetic dogs have anti–β-cell antibodies,[62] but the evidence of immune-mediated destruction of islet β cells is weak overall.[63,64] Histologic evaluation of pancreatic tissue from diabetic dogs has found a marked deficiency in β cells consistent with immune-mediated destruction.[65] However, the deficiency could be due to other causes such as persistent hyperglycemia[66] or chronic pancreatitis.

(3) Type 1 DM has been diagnosed in an adult alpaca.[67]

b. Type 2 DM: insulin resistance with inadequate compensatory insulin secretory response (insulin-resistance DM); previously called non–insulin-dependent DM, type II DM, or adult-onset DM

(1) This form of DM is most common in cats and is caused by defects in insulin secretion and postinsulin receptor defects in target cells,[61] the two major criteria for type 2 DM.

(2) In cats, as in people, evidence has supported that amylin (islet amyloid polypeptide hormone) normally produced by pancreatic β cells is often involved in the disorder.[68] When amylin is overproduced, it accumulates in pancreatic islets (pancreatic amyloidosis) and damages β cells. The hypothesis that extracellular amyloid deposition causes DM through general islet damage is doubted because functions of other islet cells are not decreased.[69] However, intracellular amyloid fibrils produced by β cells may play an important role by triggering apoptosis of β cells.[70] Whether amylin promotes insulin resistance in target cells is unclear.

(3) Glucotoxicity: *Glucotoxicity* (glucose toxicosis) is the structural and functional damage to β cells and insulin's target tissues caused by chronic hyperglycemia. It may contribute to several types of DM in cats and dogs by down-regulating GLUTs on β cells and promoting production of reactive oxidative species in β

cells. With fewer transporters, hyperglycemia is not recognized by β cells, and thus there is not appropriate insulin secretion.[71] Reactive oxidative species can reduce insulin secretion and promote β-cell apoptosis.[72] Correcting hyperglycemia may allow islets to regain function and even resolve the clinical diabetic state.

(4) Lipotoxicity: Persistent increases in saturated FAs may lead to impaired β-cell response to hyperglycemia, suppress proinsulin synthesis, and promote apoptosis of β cells through several complicated metabolic and inflammatory paths. This lipotoxicity also contributes to development of DM.[73,74]

(5) Obesity is associated with an increased incidence of DM in cats (and people).[75] As cats changed from a lean state to an obese state, they developed glucose intolerance and lower GLUT4 expression.[76] The results suggest that decreased GLUT4 expression occurs in obese cats before they develop clinical DM. Alterations in blood FA, leptin, and glucagon concentrations may also be contributing factors to the feline diabetic state.[61] Even though there are alterations in glucose tolerance and insulin secretion in obese dogs, obesity does not appear to cause clinical DM in dogs.[61,77]

c. Pancreatic DM: Any pancreatic disease (e.g., pancreatitis, partial pancreatectomy) may damage enough β cells to cause DM. This association is common in dogs and probably occurs in cats more frequently than commonly recognized.

(1) Although difficult to determine whether pancreatitis is secondary to DM or responsible for it, published reviews suggest that about 30 % of canine DM cases are caused by end-stage chronic pancreatitis (e.g., < 20 % functional pancreatic tissue).[78] In one study, 13 % of the diabetic dogs were diagnosed as having pancreatitis.[79] Of 62 cases of clinical canine DM, 45 dogs had increased pancreatic lipase immunoreactivity; 31 of those dogs had ultrasonographic evidence of pancreatitis.[80] Of 127 cases of canine ketoacidotic DM, 52 had ultrasonographic or histologic evidence of acute pancreatitis.[81] DM has also been recognized in a 3-mo-old Labrador retriever with pancreatic acinar atrophy and associated exocrine pancreatic insufficiency.[82]

(2) The relationship between feline pancreatitis and DM has not been clearly defined for a few reasons: (i) feline pancreatitis has been difficult to diagnose antemortem, (ii) cats with DM might have pancreatitis in addition to other diseases affecting hepatobiliary, renal, or intestinal tissues, and (iii) glucotoxicity may damage enough pancreatic cells to initiate pancreatitis.[83] Based on postmortem examination of 37 cats with clinical DM, 17 had chronic pancreatitis and two had acute or subacute pancreatitis.[84]

(3) Partial pancreatectomy (e.g., for excision of insulinomas in dogs or cats) may cause transient hyperglycemia or persistent DM.[85]

(4) DM is rarely reported in cattle[86] and horses.[87]

d. Other endocrine DM

(1) Hypersomatotropism (acromegaly) in dogs and cats: Hypersomatotropism is a pathologic state due to excess production of GH (somatotropin). Excess GH creates insulin resistance by causing insulin receptor and postreceptor defects.[88,89] GH promotes hyperglycemia by two major mechanisms: (i) it stimulates gluconeogenesis by hepatocytes, and (ii) persistent hyperglycemia promotes hyperinsulinemia, which eventually leads to down regulation of insulin receptors in target tissues and thus insulin resistance. In the presence of

insulin in the liver, GH promotes the production of IGF-1. Excess IGF-1 causes the soft tissue and bone lesions of acromegaly.

(a) In cats, up to one third of clinical DM cases are due to hypersomatotropism,[90,91] which may occur without DM and progress to it through a state of glucose intolerance.[92] The excess GH production is typically due to a functional pituitary adenoma;[89] the neoplasm may also produce ACTH, melanocyte-stimulating hormone, or follicle-stimulating hormone.[92] A diagnosis of hypersomatotropism is based on finding increased [GH] if a validated assay is available, sufficiently increased [IGF-1], and evidence of a pituitary neoplasm based on computed tomography or magnetic resonance imaging. Increased [IGF-1] may not be found prior to insulin therapy because IGF-1 production by hepatocytes is insulin dependent and some cases of hypersomatotropism do not have clinical DM when first diagnosed. When a plurihormonal adenoma produces GH and ACTH, a cat may have concurrent hypersomatotropism and hyperadrenocorticism.[93] GH is produced by feline mammary glands, but it does not appear to enter blood.[57]

(b) In contrast to cats, canine hypersomatotropism and associated DM or glucose intolerance rarely is due a somatotropinoma.[94,95] The increased GH production is typically due to a progestin, either an exogenous progesterone-like compound, e.g., medroxyprogesterone,[96] or endogenous progesterone, e.g., during pregnancy or the corpus luteum-phase of estrus.[57,97–100] A high incidence of diestrus-associated DM has been reported in elkhounds, particularly when older.[101,102] Progestin stimulates canine mammary glands to produce excess GH; however, there may not be sufficient alterations to create DM. Similarly, canine mammary neoplasms may directly produce GH that promotes glucose intolerance.[103] As in the cat, hypersomatotropism-induced DM is documented by finding increased [GH] or [IGF-1]. There also are reports of excess GH production in dogs with primary hypothyroidism; such dogs may or may not have clinical DM.[104,105]

(2) Glucagonoma: A glucagonoma is a pancreatic α-cell neoplasm that secretes glucagon. Excess glucagon antagonizes insulin activity by stimulating gluconeogenesis and inhibiting glucose utilization and storage; glucose metabolism may be altered sufficiently to create DM.[106] This form of insulin-resistant DM is rare in domestic mammals. It may be accompanied by superficial necrolytic dermatitis (necrolytic migratory erythema).

(3) Hyperadrenocorticism

(a) Excess cortisol antagonizes insulin activity by stimulating gluconeogenesis, creating insulin resistance, and potentially inducing apoptosis of pancreatic β cells (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section III.B.1.c). In one study, 23 % of the diabetic dogs were diagnosed as having hyperadrenocorticism.[79] In another study, 14 % of dogs with hyperadrenocorticism had concurrent DM; DM occurred with pituitary-dependent hyperadrenocorticism more frequently than with functional adrenocortical neoplasms.[107] Most cats with hyperadrenocorticism (77–80 %) also have DM.[93,108]

(b) DM and hyperadrenocorticism are sometimes considered two different diseases, but both are pathophysiologic states that develop from various specific diseases (e.g., pituitary-dependent hyperadrenocorticism). Although concurrent pathophysiologic states can develop from different concurrent diseases, data support the concept that hyperadrenocorticism leads to DM.

(4) Hyperpituitarism: A diabetic state can be created through excess secretion of GH or ACTH (which stimulates cortisol production).
   (a) A pituitary adenoma that was producing ACTH created an insulin-resistant diabetic state in a cat. The adenoma also produced α-melanocyte–stimulating hormone.[109]
   (b) Pituitary adenomas, one producing ACTH and one producing GH, were found in a cat that had clinical signs and laboratory data indicative of hyperadrenocorticism and DM.[110]
   (c) Some horses with pituitary adenomas secondary to PPID may be hyperglycemic because of secretion of ACTH and the resultant excess production of glucocorticoid hormones.[111] Although rare, most equine DM cases are probably associated with PPID and insulin resistance.[112]

(5) Hyperthyroidism
   (a) Studies in cats suggest that hyperthyroidism creates a state of glucose intolerance that might produce or contribute to hyperglycemia.[113,114] The mechanism is not known but evidence supports roles for defective insulin secretion and insulin resistance.[115,116] Only approximately 5 % of hyperthyroid cats are diagnosed with DM, and it is not clear if DM develops independently or if hyperthyroidism is a significant contributor.[117,118]
   (b) Serum [fructosamine] is expected to be increased in cats with DM. However, it may not be increased in diabetic hyperthyroid cats because plasma proteins have a shorter circulating half-life in hyperthyroidism and thus less time is available for them to become glycated.[119]

(6) Hypothyroidism: Some dogs with untreated hypothyroidism have DM (5 of 50 in one study); a direct link between hypothyroidism and DM was not established in the reported cases.[120] The concurrent finding of hypothyroidism and hyperglycemia may, in some cases, be because of a genetic predisposition to both thyroid disease and DM.[121] However, hypothyroidism may promote DM through increased insulin resistance caused by increased [GH] and increased [IGF-1].[122] In one study of hypothyroid dogs that had increased [fructosamine], the hypothyroid dogs were not hyperglycemic; increased [fructosamine] may have been caused by increased half-lives of plasma proteins.[123]

(7) Pheochromocytoma: Catecholamines (epinephrine and norepinephrine) secreted in excess by pheochromocytomas stimulate glycogenolysis and promote GH release (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section I.A.6). Plasma concentrations of catecholamines and their metabolites (metanephrine, normetanephrine) are used to diagnose pheochromocytomas (see Chapter 18). Available glycogen stores may limit the severity of hyperglycemia. Hyperglycemia in pheochromocytoma cases has been reported in dogs[124] and horses.[125]

(8) Bovine milk fever: Hyperglycemia occurring with milk fever may be partially caused by Ca^{2+} deficiency; Ca^{2+} is involved in the cleavage of proinsulin to insulin.[126,127] Other physiologic responses in stressed cows may contribute to the hyperglycemia via catecholamines, cortisol, or both.

(9) Canine aminoaciduric hypoaminoacidemic hepatopathy syndrome (canine hepatocutaneous syndrome when superficial necrolytic dermatitis is present): Hyperglycemia with a clinical diagnosis of DM has been reported in 20–30 % of dogs with this syndrome, being present at diagnosis or developing later.[128] The pathogenesis of hypoaminoacidemia and hyperglycemia have not been established, but potential roles for glucagon and GLP-1 have been reported.[128] Ultrasonographically, the liver typically has a honeycomb or Swiss cheese appearance. Hepatic histologic lesions in affected dogs typically include marked glycogen and lipid vacuolation of hepatocytes, nodular hyperplasia, and various degrees of fibrosis. Increased serum activities of alkaline phosphatase and alanine aminotransferase, anemia, microcytosis, and hypoalbuminemia are common laboratory features. About three-fourths of the affected dogs develop superficial necrolytic dermatitis. Glucagonomas also cause superficial necrolytic dermatitis but without the hepatic lesion.[129]

e. Drug-induced DM: persistent hyperglycemia (2 d or more) associated with use of a drug

(1) Glucocorticoids[130] (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section III.B.1.c)

(2) Thyroid hormones: Studies in cats suggest that hyperthyroidism creates a state of insulin resistance, but the mechanism is not known.[113,114] In a study in which thyroxine was given to create experimental hyperthyroidism in dogs, data indicated that glucose-induced hyperglycemia was prolonged because of defective insulin secretion.[115] Decreased insulin secretion and insulin resistance may both promote hyperglycemia.[116]

(3) Megestrol acetate: As a steroid that can bind to the glucocorticoid receptor, it promotes gluconeogenesis; as a progestin, it stimulates release of GH (see Glucose Concentration In Serum, Plasma, or Whole Blood, Section III.B.2.d.(1)(b)). Both mechanisms probably occur in dogs, but perhaps only the latter occurs in cats.[131]

(4) Medroxyprogesterone acetate and proligestone have sufficient glucocorticoid activity to suppress the hypothalamic–pituitary–adrenal axis, induce a steroid hepatopathy, and, with long-term treatment, cause iatrogenic hyperadrenocorticism and DM.[132] These progestins also stimulate release of GH.

f. Infectious DM

(1) Cattle infected with bovine viral diarrhea virus can develop DM that appears to result from damage to β cells.[133,134]

(2) Sepsis: An early effect of endotoxemia is insulin resistance and possibly impaired insulin secretion by β cells resulting in hyperglycemia. Hypoglycemia may develop later. There are several hormonal and cellular responses in sepsis (and noninfectious inflammation) that alter glucose metabolism.[135] Because animals with DM are considered more susceptible to infections (especially urinary), sepsis could be the cause or result of DM.

g. Hyperammonemia (primarily horses and cattle): Hyperammonemia occurs in horses and cattle for a variety of reasons (Chapter 13), and hyperglycemia may be a concurrent finding.[136,137] Hyperglycemia may develop secondary to catecholamine or glucocorticoid release, or because NH_4^+ stimulates the release of glucagon, which then promotes gluconeogenesis and reduces glucose utilization by tissues.[138] If mediated by glucocorticoids or NH_4^+, hyperglycemia would depend on glucose production by hepatocytes, and thus hyperglycemia would be more likely when the hyperammonemia is caused by disorders other than hepatic insufficiency (e.g., excess NH_4^+ production in intestine, urea toxicosis, and ammonia forage toxicosis). Certain inborn errors of metabolism, including urea cycle deficiencies, have caused hyperammonemia and hyperglycemia in people.

h. Genetic DM
   (1) An inherited form of DM occurred in keeshonden. The onset of the disorder was frequently before 6 mo of age and was caused by β-cell hypoplasia.[139]
   (2) A probable familial insulin-dependent DM was reported in adult Samoyed dogs. The pathogenesis of the diabetic state was not established.[140]
   (3) Certain breeds of dogs (e.g., Alaskan malamute, Finnish spitz, standard and miniature schnauzer, miniature poodle, Australian terrier, fox terrier, Tibetan terrier, Cairn terrier, and dachshund) and cats (Burmese, Tonkinese, Norwegian Forest) have a higher incidence of DM, but a genetic pathogenesis is not established in those breeds.[61,141–144]
   (4) Congenital defects in pancreatic development might have caused DM in a 6-wk-old kitten.[145]

i. Anti-insulin antibodies: Dogs with anti-insulin antibodies prior to insulin treatments have been reported, but in low number and without case details.[61,146,147] In contrast to autoantibodies, diabetic dogs treated with bovine, porcine, and human insulin have developed anti-insulin antibodies, but with no clear impact on glycemic control to date.[148,149] A diabetic cat that was refractory to treatment was positive for anti-(human insulin) antibodies, but it was not tested prior to treatment and the anti-insulin antibodies were not clearly tied to refractoriness given that they persisted after clinical control.[150]

3. Pharmacologic or toxicologic hyperglycemia: transient hyperglycemia associated with administration or ingestion of a drug or toxicant (persistent use of some agents may produce a disorder that fulfills diagnostic criteria for DM) (see Table 14.3)
   a. See the previous section for hyperglycemic agents that may cause transient hyperglycemia or DM when persistently present: glucocorticoids, glucagon, thyroxine, and progestins (megestrol acetate, medroxyprogesterone acetate, and proligestone).
   b. Transient hyperglycemia may be caused by oral or intravenous glucose (dextrose) entering plasma faster than it is utilized, stored, or excreted.
   c. Ethylene glycol is converted to aldehydes that inhibit glycolysis and the Krebs cycle, and thus it may indirectly stimulate gluconeogenesis in hepatocytes.[151,152] Concurrently, stress-induced cortisol release may contribute to hyperglycemia.
   d. α_2-adrenoceptor agonists: Xylazine and detomidine bind to α_2-adrenergic receptors on β cells, resulting in the inhibition of insulin release and thus reduction in glucose utilization by hepatocytes, myocytes, and adipocytes.[153] Dexmedetomidine and presumably similar drugs also promote hyperglycemia. Ingestion of amitraz, a pesticide with α_2-adrenergic effects, has caused hyperglycemia, decreased mentation,

and bradycardia in dogs.[154] Effects of these agonists may be reversed by α_2-adrenoceptor antagonists such as atipamezole and yohimbine.

e. Intravenous octreotide, a somatostatin analog, induces hyperglycemia through its binding to β-cell receptors, thus inhibiting the release of insulin.[155]

f. Propranolol and other β-adrenergic blockers inhibit insulin release from β cells. Additionally, propranolol may create a state of insulin resistance.[156]

g. Albuterol (salbutamol) toxicity causes hyperglycemia in many affected dogs.[157] Although it increases insulin secretion by stimulating β2-adrenoceptors on pancreatic β cells, it apparently has a greater impact on increasing gluconeogenesis in hepatocytes and glycogenolysis in hepatocytes and skeletal muscle. Hyperglycemia may be amplified in diabetics. Hyperlactatemia, hypokalemia, increased [FA], and hypotension are also commonly present.

h. Insulin (rebound hyperglycemia or posthypoglycemic hyperglycemia, Somogyi effect): Usually, hyperglycemia developing in diabetics after insulin-induced hypoglycemia is related to waning or insufficient insulin. In rare cases, hyperglycemia after insulin administration might reflect a state of insulin resistance caused by hypoglycemia-induced increases in counterregulatory hormones including glucagon, epinephrine, cortisol, and GH (the Somogyi effect or theory), but this has not been documented. Such a post-insulin hyperglycemic response would be controlled by insulin release in healthy (nondiabetic) animals. Posthypoglycemic hyperglycemia has been documented in ~10–25 % of insulin-treated diabetic cats for which glucose curves were available to evaluate, but in only ~2 % of their curves, and with evidence of insulin resistance in only 4 of 10,767 curves in one study.[158,159]

IV. Hypoglycemia

A. Hypoglycemic disorders are caused by increased glucose utilization by tissues, decreased glucose production, or both.

B. A diagnostician should always be alert to the possibility of a mild to extreme spurious hypoglycemia created by the following: (i) in vitro glycolysis in WBCs, RBCs, platelets, and possibly bacteria, particularly with delayed analysis (common), (ii) bromide interference (in samples from patients receiving KBr) in glucose oxidase assays, or (iii) interference by paraproteinemia in photometric hexokinase assays (rare). The consumption of glucose by cells may increase with increased cell concentrations or because of increased metabolic activity in WBCs (e.g., malignant blast cells or after stimulation by granulocyte colony–stimulating factor).[160] A WBC needs more glucose than a RBC, but there are about 1000 times as many RBCs as WBCs in health. Marked rubricytosis would also probably cause increased glucose consumption.[161]

C. Disorders and pathogeneses (Table 14.5)

1. Pathologic hypoglycemia

a. Increased insulin secretion

(1) Functional pancreatic β-cell neoplasm (insulinoma), a pancreatic neuroendocrine neoplasm

(a) Hyperinsulinism causes decreased glucose production by hepatocytes and increased glucose utilization by hepatocytes, myocytes, and adipocytes. Insulinomas may cause persistent or sporadic hypoglycemia of sufficient severity to cause weakness and seizures. With sporadic hypoglycemia, a prolonged fast (up to 72 h) may be needed to document hypoglycemia.

Table 14.5. Disorders and conditions that cause hypoglycemia
Pathologic hypoglycemia
Increased insulin secretion
Functional pancreatic β-cell neoplasm (insulinoma)
Other pancreatic neuroendocrine neoplasms
Non-insulinoma pancreatogenous hypoglycemia (acquired nesidioblastosis)
Idiopathic hyperinsulinemic hypoglycemia
Xylitol toxicosis (dogs)
IGF-2-induced paraneoplastic hypoglycemia (IGF-2-omas)
Decreased insulin antagonists
Hypoadrenocorticism (decreased cortisol)*
GH deficiency
Hypopituitarism (decreased ACTH, GH, or both)
Decreased gluconeogenesis
Hepatic insufficiency/failure: acquired, congenital*
Hypoadrenocorticism (decreased cortisol)*
Neonatal or juvenile hypoglycemia
Starvation, severe malnutrition, milk-deprived neonates
Decreased glycogenolysis
Glycogen storage diseases including glycogen branching enzyme deficiency in foals and kittens (rare)
Increased glucose utilization
Lactational hypoglycemia (spontaneous bovine ketosis)*
Exertional hypoglycemia (hunting dogs, endurance horses)
Other pathologic hypoglycemias with uncertain or multiple pathogeneses
Sepsis, especially with endotoxemia*
Portosystemic shunts
Heatstroke (heat-induced illness)
Pregnancy hypoglycemia
Refeeding syndrome (cats)
Amphetamine toxicity
Malonic aciduria in Maltese dogs
Methylmalonic aciduria
Lipid storage myopathy (foal)
Renal glucosuria due to acute tubular nephrosis
Pharmacologic or toxicologic hypoglycemia: insulin, sulfonylurea compounds (glipizide, glyburide), ethanol, metformin, amanitin-containing mushrooms)

* A relatively common disorder, condition, or mechanism

Note: Delayed analysis of blood samples, sometimes compounded by failure to separate serum or plasma from blood cells, may cause falsely low [glucose] because of glucose utilization by cells. Marked leukocytosis and erythrocytosis may accelerate glucose consumption. Bromide ions cause falsely low [glucose] when using the i-STAT instrument or method. Rarely, paraproteins cause pseudohypoglycemia with certain assays (see text). Because of substantial differences in results generated by different methods and instruments (see Glucose Concentration in Serum, Plasma, or Whole Blood, Sections II.C and D), applicable RIs should be used to identify hypoglycemia.

(b) Insulinomas are reported uncommonly in dogs, rarely in cats,[162] and relatively commonly in ferrets. Rarely, an insulin-secreting neoplasm can be part of multiple endocrine neoplasia type 1 or a similar concurrence of multiple endocrine neoplasms.[163]

(c) Documentation of hyperinsulinism requires a measured [IRI] and increased IRI:G ratio (see Immunoreactive Insulin (IRI) Concentration, Sections II and V). An [IRI] that is increased or within RI when there is concurrent hypoglycemia represents an inappropriate [IRI] and is supportive of an insulinoma.[164,165]

(2) Other pancreatic neuroendocrine neoplasms: A retrospective study found 55 cases of hypoglycemia associated with histologically diagnosed pancreatic neuroendocrine neoplasms. The neoplastic tissues were positive for IGF-2 and synaptophysin. Concurrent hypoinsulinemia supported IGF-2 as the mediator of hypoglycemia.[166]

(3) Noninsulinoma pancreatogenous hypoglycemia (acquired nesidioblastosis): This acquired nonneoplastic hyperinsulinemic condition is characterized histologically by a focal or diffuse hypertrophy and hyperplasia of functional islet β cells. It has been described in a cat and a dog.[167,168] In the canine case, hypoglycemia resolved after partial pancreatectomy. The histologic lesion of islet neogenesis from pancreatic ductal epithelium can be present without hyperinsulinemia and associated hypoglycemia; this has been reported as nesidioblastosis in young dogs, a Simmental calf, and cattle with double muscling.

(4) Idiopathic hyperinsulinemic hypoglycemia: Congenital hyperinsulinism occurs in children with any of several mutations. It was suspected in a young shiba inu that was presented with seizures; the dog was successfully managed for at least 2.5 yr.[169] Suspected congenital hyperinsulinemic hypoglycemia was also reported in two young dogs that had bartonellosis, one with no histologic abnormalities of the pancreas and one with mild to moderate islet cell hyperplasia.[170]

(5) Xylitol toxicosis

(a) *Xylitol* is a five-carbon sugar alcohol (polyol) that is used as a sugar substitute (alone or with aspartame) in numerous products including some candies, baked goods, jams and jellies, peanut butters, yogurts, ice creams, chewing gums, toothpastes, mouthwashes, vitamins, medications, and products not intended for ingestion.[171] In dogs, it is a potent stimulant for release of insulin, which then promotes increased glucose uptake and utilization, and thus hypoglycemia.[172,173] When it is given per os, xylitol causes a greater release of insulin than when glucose is given per os. Xylitol stimulates insulin release in cattle and goats, but not in people and horses. It does not cause hypoglycemia in cats.

(b) Many canine cases of hypoglycemia due to xylitol toxicosis have been described.[174] The dogs may also develop acute hepatic toxicosis with a coagulopathy and increased serum or plasma activities of alanine transaminase (ALT) and aspartate transaminase (AST), as well as hypophosphatemia and hypokalemia related to insulin-induced shifts.[175] In experimental toxicosis, the hyperbilirubinemia was mostly of indirect bilirubin, supporting either a hemolytic effect or impaired bilirubin uptake by hepatocytes.

b. IGF-2-induced paraneoplastic hypoglycemia (IGF-2-omas) (non–islet-cell tumor hypoglycemia)[176]
   (1) A variety of benign or malignant, epithelial or nonepithelial, non–β-cell neoplasms in people and domestic mammals have been shown or suspected to cause hypoglycemia (typically fasting) via secretion of either IGF-2 or incompletely processed forms of IGF-2 (big IGF-2) that bind to insulin receptors and IGF receptors. Alterations in IGF-2 and its binding proteins can increase the number of free IGF-2 molecules and decrease the size of IGF-2–protein complexes such that there is greater diffusion and tissue activity. Because of these factors, hypoglycemia may be mediated by IGF-2 without an increased serum [IGF-2].
   (2) Like insulin, IGF-2 promotes hypoglycemia by increasing glucose uptake and consumption by skeletal muscle, inhibiting hepatic glycogenolysis and gluconeogenesis, and decreasing glucagon and GH secretion (thus impairing the physiologic responses to hypoglycemia). IGF-2 also inhibits ketogenesis, decreases lipolysis in adipocytes, and suppresses insulin secretion, which can cause postprandial hyperglycemia. Complete excision of an IGF-2-oma corrects the fasting hypoglycemia, decreases the [IGF-2] if it was increased, and increases the previously depressed concentrations of glucagon, GH, IGF-1, and insulin.
   (3) Neoplasms with evidence supporting classification as IGF-2-omas in veterinary medicine include canine hepatocellular carcinoma,[177] canine mammary carcinoma,[178] canine gastric and splenic leiomyomas,[179,180] canine jejunal leiomyosarcoma,[177] and possibly equine renal carcinoma.[181] IGF-2 may have mediated hypoglycemia associated with other non–β-cell neoplasms reported in veterinary species: leiomyomas, leiomyosarcomas, hepatoma or hepatocellular carcinomas, cholangiocarcinoma, mesothelioma, and gastrointestinal stromal tumor.[182–190]

c. Decreased insulin antagonists
   (1) Hypoadrenocorticism
      (a) When hypoglycemia is present in hypoadrenocorticism, it is probably because of hypocortisolemia, thus increased insulin sensitivity, increased glucose consumption, and decreased gluconeogenesis.[191,192] The hypothesis that the hypoglycemia is a response to hyperkalemia-stimulated insulin release was not supported by data from 11 canine cases.[193]
      (b) Mild to moderate hypoglycemia is found in up to 20 % of dogs with primary or secondary hypoadrenocorticism;[194,195] similar or greater percentages have been described in atypical primary hypoadrenocorticism.[196,197] Hypoglycemia has also been present in the uncommon feline cases of primary and secondary hypoadrenocorticism.[198,199]
   (2) GH deficiency: Reduced GH activity promotes increased insulin sensitivity and thus has the potential to cause hypoglycemia; hypoglycemia is a clinical feature of GH deficiency in people. Hypoglycemia was reported in a kitten with juvenile hyposomatotropism and a decreased [IGF-1],[200] and in a Chihuahua puppy with congenital hyposomatotropism.[201]
   (3) Hypopituitarism: Hypoglycemia is found in people with hypopituitarism and could be caused by decreased secretion of GH, ACTH (and thus less cortisol production), or both. Hypoglycemia was described in a dog with partial

pituitary deficiency and evidence of hypocortisolemia and decreased [GH].[202] When hypophysectomy is used to treat feline hypersomatotropism, hypoglycemia develops in some cats once exogenous insulin is discontinued; this might involve hyperinsulinism secondary to β-cell hyperplasia that developed during prior GH-induced insulin resistance.[203]

d. Decreased gluconeogenesis
   (1) Hepatic insufficiency: With marked reduction in functional hepatic mass because of congenital or acquired disorders, there are too few hepatocytes to maintain fasting normoglycemia. Other evidence of hepatic disease or insufficiency is expected, e.g., hypoalbuminemia, decreased [urea nitrogen], increased [bile acid], increased serum activities of enzymes released from hepatobiliary cells.
   (2) Hypoadrenocorticism (see immediately preceding Section IV.C.1.c.(1))
   (3) Neonatal or juvenile hypoglycemia: This neonatal canine disorder occurs in toy and miniature breeds and may be caused by hepatic immaturity and insufficient gluconeogenesis relative to metabolic rate and glucose consumption.[204]
   (4) Starvation, severe malnutrition, or milk-deprived neonates: With chronic depletion of body fuels (including proteins and fats), gluconeogenesis may not maintain normoglycemia. A starved state may be caused by lack of food intake, maldigestion, or intestinal malabsorption. Generally, physiologic pathways maintain blood glucose at the expense of other body fuels, and thus animals with this form of hypoglycemia are expected to be markedly underweight or emaciated. Even then, hypoglycemia is uncommon.[205] Energy stores (glycogen, fat) are limited in neonates, so diminished intake of milk may cause or contribute to hypoglycemia.

e. Decreased glycogenolysis: Congenital deficiencies of enzymes needed for glycogenesis or glycogenolysis may contribute to hypoglycemia and accumulation of glycogen in cells (glycogen storage diseases).[206,207] These include glycogen branching enzyme deficiency in young quarter horses, paint horses, and Norwegian forest cats.[208,209] Impediments to nursing promote hypoglycemia in affected animals.

f. Increased glucose utilization
   (1) Lactational hypoglycemia (spontaneous bovine ketosis): During marked milk production (especially in very productive cows), there is a huge need for glucose in mammary glands. The cows become hypoglycemic if gluconeogenesis cannot meet demand. Ketosis develops secondarily because of enhanced fatty-acid catabolism.
   (2) Exertional hypoglycemia: Hunting dogs and endurance horses may become hypoglycemic because glycolysis consumes glucose faster than it is replaced by either glycogenolysis or gluconeogenesis. In contrast, catecholamine and other factors tend to cause hyperglycemia in exercised animals.
   (3) Extreme leukocytosis: Some leukemias and inflammatory states (see Chapter 2) have been reported to cause hypoglycemia because of increased glycolysis. Depending on sample handling, in vitro glycolysis could contribute to effects of in vivo glycolysis. Increased glucose consumption may occur because of increased numbers of WBCs or because of increased metabolic activity in WBCs (e.g., malignant blast cells or after stimulation by granulocyte colony–stimulating factor).[160]

g. Other pathologic states causing hypoglycemia through uncertain or multiple mechanisms
   (1) Sepsis (especially endotoxemia)
      (a) Hypoglycemia is probably due to increased utilization by tissue (e.g., WBCs) and decreased glucose production. Glucose consumption by organisms is likely not a significant contributor in most infections. Endotoxins have been shown to induce hypoglycemia. Factors contributing to endotoxemic hypoglycemia are thought to include increased insulin sensitivity and cytokine-mediated reduction in the synthesis and activity of enzymes important for hepatic gluconeogenesis.[210] Decreased use of lactate for gluconeogenesis may promote hyperlactatemia, as may epinephrine-stimulated aerobic glycolysis and a shift to anaerobic glycolysis in tissues with decreased perfusion (see Chapter 9, Lactate Concentration).
      (b) If there are numerous organisms, they might contribute to the hypoglycemia.[211]
         (i) Severe hemotropic mycoplasma infections in lambs, crias, and pigs have been associated with severe hypoglycemia and hyperlactatemia, sometimes with clinical signs attributable to hypoglycemia (e.g., seizures).[211,212] In vitro glucose metabolism by the organisms can contribute substantially to these changes. Porcine RBCs use inositol, not glucose, for energy, so hypoglycemia cannot be attributed to RBC consumption of glucose in pigs.
         (ii) Refractory hypoglycemia was found in a dog infected with *Trypanosoma congolense*. Clinical findings and related literature indicate that consumption of glucose by trypanosomes contributes to hypoglycemia.[213]
         (iii) Hypoglycemia is common in foals with Tyzzer's disease caused by *Clostridium piliforme*.[214] In addition to the effects of sepsis, widespread hepatic necrosis could cause decreased gluconeogenesis.
   (2) Portosystemic shunts: Hypoglycemia occurs in a minority of fasted dogs with congenital portosystemic shunts and can also develop postoperatively.[215,216] Impaired hepatic function likely contributes to the hypoglycemia.
   (3) Heatstroke (heat-induced illness): Hypoglycemia is common in dogs with heat-induced illness.[217] Experimental studies in dogs suggest that increased use of respiratory muscles to promote cooling may contribute to increased energy needs and glucose consumption (exertional hypoglycemia).[218] Hepatic dysfunction and sepsis are regarded as other potential contributors to hypoglycemia.
   (4) Pregnancy hypoglycemia
      (a) Hypoglycemia (with or without ketosis) related to pregnancy occurs in bitches, but uncommonly.[219] Experimental studies indicated the hypoglycemia is probably due to inadequate glucose production from glycogenolysis concurrent with increased glucose consumption by uteroplacental-fetal tissues.[220] Ketosis occurs because of excessive breakdown of FAs. Decreased appetite of late pregnancy bitches can lead to ketotic hypoglycemia.

(b) Pregnancy-associated hypoglycemic and ketotic states in ruminants include pregnancy toxemia in sheep and periparturient bovine ketosis in cattle (see Ch. 9, β-Hydroxybutyrate (BHB) and Acetoacetate (AcAc) Concentration, Section III).

(5) Refeeding syndrome in cats: Refeeding syndrome caused by the reintroduction of enteral or parenteral nutrition to malnourished or starved cats (or people) can include hypoglycemia along with the more consistent findings of hypophosphatemia and hypokalemia.[221] Factors contributing to hypoglycemia may include a strong insulin response to nutrients coupled with diminished glycogen stores and impaired gluconeogenesis. Concurrent factors, e.g., sepsis, may also contribute. However, hyperglycemia is also common during hospital management of these cats.

(6) Amphetamine toxicosis has caused hypoglycemia in dogs and cats.[222] Increased glucose consumption secondary to tremors, seizures, and panting in response to hyperthermia likely contribute. Amphetamines have been shown to directly stimulate insulin release and cause hypoglycemia in mice;[223] the response was variable in rats but might contribute to hypoglycemia in dogs and cats.

(7) Malonic aciduria in Maltese dogs: Dogs with this metabolic disorder had marked hypoglycemia, but the specific enzymatic defect was not established. The dogs did not have malonyl-coenzyme A (CoA) decarboxylase deficiency.[224] Decreased gluconeogenesis probably contributed to the hypoglycemia, because increased [malonyl-CoA] inhibits pyruvate carboxylase, an enzyme in a gluconeogenesis pathway.

(8) Methylmalonic aciduria: Hypoglycemia has been recognized in people with methylmalonic aciduria, which is caused by impaired activity of methylmalonyl CoA mutase, a cobalamin-dependent enzyme. Hypoglycemia has also been reported in dogs with methylmalonic aciduria secondary to a cobalamin deficiency.[225] Although poor intake, seizures, and young age may have contributed to hypoglycemia in these dogs, experimental studies indicate that increased methylmalonic acid interferes with mitochondrial transport of malate, α-oxoglutarate, and isocitrate, and thus interferes with gluconeogenesis.[226]

(9) Lipid storage myopathy: The affected neonatal paint filly foal had persistent hypoglycemia and weakness with other findings similar to human multiple acyl-CoA dehydrogenation deficiency, a group of conditions in which there is a defect in FA and amino acid oxidation.[227]

(10) Renal glucosuria due to acute tubular nephrosis: Various commercial dog treats were contaminated with melamine or its analogs; the compounds caused proximal tubular nephrosis, which caused glucosuria.[228] In a retrospective study of such cases, 22 of 96 dogs had concurrent glucosuria and hypoglycemia at presentation.[229] Poor resorption of glucose by proximal tubules contributed to the hypoglycemia, but many dogs were not eating and thus had decreased intake of carbohydrates.

2. Pharmacologic or toxicologic hypoglycemia

a. Insulin

(1) An overdose of insulin in a diabetic animal may cause hypoglycemia because of the excess utilization of glucose and decreased gluconeogenesis. The amount of insulin that diabetics needs depends on food intake, physical activity, concurrent conditions, and other factors.[230] Decreased glomerular filtration rate reduces insulin clearance.

(2) Secretive and malicious administration of insulin to a horse has been reported.[231]

b. Sulfonylurea compounds (glipizide and glyburide): These drugs, sometimes referred to as oral hypoglycemic agents, directly stimulate insulin secretion and may also improve cellular responses to insulin.

c. Ethanol: Ethanol oxidation generates reduced nicotinamide adenine dinucleotide (NADH). When there is an acute excess of ethanol, an increased ratio of NADH to nicotinamide adenine dinucleotide (NADH:NAD) blocks gluconeogenesis and may cause hypoglycemia, particularly with fasting or anorexia.[232]

d. Metformin: Accidental consumption of metformin (dimethylbiguanide), an oral antihyperglycemic agent used to treat human type 2 DM, has caused marked hypoglycemia with acidemia and hyperlactatemia in small dogs.[233,234] Its effects vary with dose and metabolic state (e.g., healthy versus diabetic). Hypoglycemia is thought to be caused mainly by inhibition of glycerol-3-phosphate dehydrogenase and therefore hepatic gluconeogenesis from lactate and glycerol.[235]

e. Amanitin-containing mushrooms: Hypoglycemia is common and may be severe.[236] Amanitin-induced hypoglycemia has been associated with early accelerated glycogenolysis and glycogen depletion in hepatocytes; later, hepatocellular necrosis and consequently reduced gluconeogenesis likely contributes.

V. [Glucose] during insulin therapy

A. Serial blood [glucose] curve

1. Data from this procedure may be useful for initial regulation of a diabetic state, for detecting suspected hypoglycemia during therapy, or for managing poorly controlled DM.[42] Results may document resistance to exogenous insulin that occurs due to diabetogenic hormones (glucocorticoids, glucagon, catecholamines, GH) or that is associated with inflammatory states (e.g., urinary tract infection, acute pancreatitis).[60] Goals are to determine a dose that yields an acceptable [glucose] nadir (ideally ~80–150 mg/dL), and then to evaluate the duration of effect so that the insulin regimen controls [glucose] for as much of each day as is practical.

2. The procedure involves measuring [glucose] frequently between insulin injections (e.g., every 2 h in dogs, every 3–4 h in cats; the intervals vary with type of insulin).[42] Guidelines for interpreting the serial [glucose] are described by Behrend[42] and also are available via aaha.org.

3. Because there can be marked variation in the serial blood [glucose] curves from one day to the next, especially during hospitalization, a decision to alter an insulin dose should follow consideration of other clinical information.[237]

4. Problems that may be revealed by serial blood [glucose] curves include the following:

a. Insufficient insulin dose: If so, increase the dose.

b. Too short an insulin effect: If so, use longer-acting insulin or give q12h.

c. Overlap of insulin effect between insulin doses: If so, use shorter-acting insulin.

d. The Somogyi effect (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section III.B.3.h): If so, reduce the insulin dose and repeat the curve in 1 wk.

B. Continuous glucose monitoring: Systems for continuous monitoring of interstitial [glucose] provide similar information as that generated from serial blood glucose curves, but they facilitate management of patients with DM and better detect peaks and nadirs of [glucose] that may be missed by more intermittent testing (see Glucose Concentration in Serum, Plasma, or Whole Blood, II.D.4).

C. Unexpected hyperglycemia: There are several possible reasons for inadequate response to insulin treatments.
   1. There may be an insufficient insulin dosage, the insulin may have deteriorated, or the owner may be having problems injecting the insulin.
   2. The caloric intake in the diet may have increased.
   3. A state of insulin resistance may have developed because of stress, hyperadrenocorticism, infections, hypothyroidism, pancreatitis, or hypersomatotropism.

D. Unexpected hypoglycemia
   1. An insulin overdose may cause hypoglycemia.
   2. Other reasons for hypoglycemia in treated diabetic animals include decreased caloric intake (e.g., anorexia, vomiting, exocrine pancreatic insufficiency, and inflammatory intestinal disease) and excess glucose utilization by tissues (e.g., excess physical activity and increased metabolic state). The diabetic state might also be due to a transient disorder that decreased in severity or resolved.

KETOAMINES: FRUCTOSAMINE AND GLYCATED HEMOGLOBIN

I. Physiologic processes
   A. When glucose reacts nonenzymatically with a protein's amino groups, mostly at lysine or valine sites, a Schiff base is formed which then converts to a more stable ketoamine (Figure 14.2).[238]
      1. Generally speaking, fructosamines are glycated proteins. In clinical chemistry, *fructosamine* refers to ketoamines that are formed by the posttranslational irreversible nonenzymatic linking of glucose to albumin or other plasma proteins (mostly IgG); glycated albumin accounts for about 80 % of human serum ketoamines.[239] The carbon backbone of these ketoamines is identical to fructose (hence the name "fructosamine"). The half-life of fructosamine molecules is generally stated to be near 2–3 wk, but the half-life varies among species and can be altered during pathologic states.

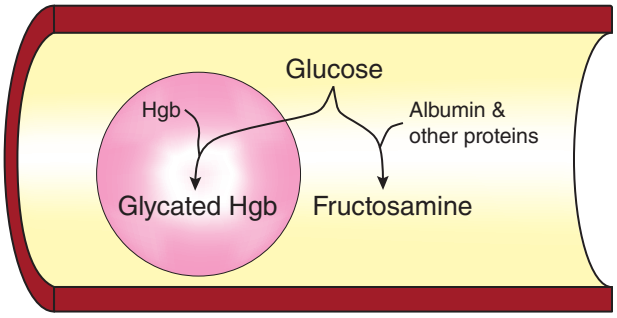


Figure 14.2. Glycated proteins (ketoamines). A small percentage of glucose transported into RBCs by facilitated diffusion nonenzymatically binds covalently to amine groups of Hgb to form glycated Hgb. The glycated Hgb persists in the RBC during the rest of the cell's life span. A small percentage of plasma glucose also nonenzymatically binds to albumin and other plasma proteins to form fructosamine. Fructosamine's half-life reflects that of the original protein molecules.

2. *Glycated Hgb* is a ketoamine formed by the nonenzymatic addition of sugars to Hgb amino groups, a process that occurs slowly and to a small degree in health as Hgb is carried for the life of a RBC. (Note: Although often called glycosylated Hgb, glycosylation refers to an enzyme-mediated addition of glucose to protein.) The half-life of glycated Hgb is generally stated to be 2–3 mo but varies with the circulating life span of RBCs (e.g., ~100 d in dogs, ~70 d in cats, and ~150 d in cattle). Hgb is a tetramer of four globular polypeptide subunits, two α globins and two non-α globins, each associated with a heme group. *Hgb A* is the most common form in adults (A for adult) with two α and two β globins. *Hgb A_1* is adult Hgb glycated on the amino terminus of a β chain, and *Hgb A_{1c}* is the subset of *Hgb A_1* with glucose as the attached sugar.

B. The formation of ketoamines in blood is positively correlated with the magnitude and duration of hyperglycemia, whereas the removal of ketoamines depends on the degradation or loss of the parent molecules (e.g., albumin or Hgb). Transient hyperglycemia, as may occur after intravenous glucose administration or in physiologic responses, does not significantly affect the concentrations of the ketoamines in dogs (nitroblue tetrazolium assay)[240] or cattle (nitroblue tetrazolium assay)[241] and should not affect ketoamine concentrations in any species. Hgb A_{1c} was shown to be a biomarker for clinical manifestations of DM in people, and it is the specific type of glycated Hgb recommended for assessment in people.[242]

II. Analytical concepts

A. [Fructosamine]

1. Serum is generally recommended. Plasma from heparinized blood may be acceptable, but values may differ from serum samples.
2. In the nitroblue tetrazolium assay, fructosamine acts as a reducing agent in an alkaline medium to generate formazan, which is detected spectrophotometrically. Nonspecific reduction occurs within the first 10 min, so measurements are taken after that time. However, other reducing agents in serum could cause false-positive results. In contrast, the fructosyl lysine oxidase assay is considered to be specific for fructosyl lysine, a specific glycated amino acid.
   a. In a comparison study, the fructosamine concentrations in canine sera determined by the nitroblue tetrazolium assay were about 3–4 times those determined by the enzymatic assay (about 100 μmol/L).[243] This study raises a concern about the validity of the [fructosamine] reported from the nitroblue tetrazolium assay.
   b. Modifications of the nitroblue tetrazolium assay to remove actions of reducing substances other than fructosamine reportedly resulted in fructosamine concentrations that were about 10 % of those generated by the original nitroblue tetrazolium assays.[244]
3. Hemolyzed, icteric, or lipemic samples can cause erroneous results with some assays.

B. Glycated Hgb concentrations or percentages

1. Many assays have been developed for glycated Hgb and specifically for Hgb A_{1c}, with considerable variation in results depending on the method. Glycated and nonglycated forms have been separated based on size or charge (e.g., various chromatographic or electrophoretic methods), chemical properties (e.g., spectrophotometric enzymatic assays), or structural differences (e.g., immunoassays), with immunoassays and high-performance liquid chromatography being most common in human medicine. Several of these methods, including reflectance spectrophotometry, column chromatography, immunochromatography (immunomigration), capillary electrophoresis, and immunoturbidometric assays for Hgb A_{1c} that use polyclonal or monoclonal antibodies

to the β-chain N-terminus, have been applied to dogs and cats.[240,245–247] There is little information and less applicability for other domestic species.

2. Results have traditionally been reported as the percentage of total Hgb that is glycated (unit: %). Reference methods that eliminated detection of Hgb–sugar complexes other than Hgb A_{1c} generated somewhat lower results and led to greater use of the SI unit: mmol/mol (millimoles of Hgb A_{1c} per mole of nonglycated Hgb A). The appropriate formulas for converting units depend on which assay standards are used. In the USA:
   a. mmol/mol = (% × 10.93) – 23.50
   b. % = (0.09148 × mmol/mol) + 2.152
3. Sample: EDTA-anticoagulated whole blood is used most commonly, but other anticoagulants may be acceptable. Stability depends on the assay method, but samples can generally remain at room temperature for a day or more and for several days to a week at 4 °C. Stability is better at –70–80 °C (many months) than at –20 °C.
4. Lipemia falsely increases or decreases results of some assays, so RBCs may need to be washed prior to analysis.[245]

III. Increased [fructosamine] and increased glycated hemoglobin percentage or concentration (Table 14.6)

A. Hyperglycemia: Increased concentrations of fructosamine or glycated Hgb occur in animals with DM. Concentrations of these glycated proteins can be used with other information to help monitor the effectiveness of controlling the diabetic state in individuals over time. Both [fructosamine] and [glycated Hgb] have been measured in diabetic dogs[244,246,248–250] and cats.[251–257] Although horses with PPID are usually not hyperglycemic, their plasma [fructosamine] has been assessed.[258]

B. Hypothyroidism
   1. Mildly increased [fructosamine] (assay type not reported) was found in hypothyroid dogs that were not considered to have DM.[120] In another study of normoglycemic normoproteinemic hypothyroid dogs, 9 of 11 had an increased [fructosamine] (nitroblue tetrazolium assay), and the concentrations decreased after initiation of thyroid hormone supplementation.[123]
   2. Based on studies in people, the increased [fructosamine] is the result of an increased albumin half-life (reduced protein turnover).[259]

C. Hyperproteinemia
   1. In theory, hyperproteinemia could cause increased [fructosamine]. However, variations in the duration of the hyperproteinemia and variations in albumin-to-globulin ratios result in variable fructosamine concentrations.[260,261]
   2. In one study, corrected [fructosamine] in hyperproteinemic animals (see upcoming Section V) did not improve the diagnostic value of [fructosamine].[260]

Table 14.6. Disorders and conditions that increase ketoamine concentrations

Fructosamine	Glycated hemoglobin
Hyperglycemia (persistent)[a]*	Hyperglycemia (persistent)*
Hypothyroidism	
Hyperproteinemia, hyperglobulinemia	

* A relatively common disorder, condition, or mechanism
[a] See Table 14.4 for causes of hyperglycemia.
Note: Anemia and RBC transfusions may reduce increases in [glycated Hgb].

Table 14.7. Disorders and conditions that decrease ketoamine concentrations

Fructosamine	Glycated hemoglobin
Hypoglycemia (persistent)[a]*	Hypoglycemia (persistent)*
Hypoproteinemia or hypoalbuminemia	Anemia (see the text for variables)
Hyperthyroidism	

* A relatively common disorder, condition, or mechanism
[a] See Table 14.5 for causes of hypoglycemia.

3. Increased fructosamine concentrations were reported in two dogs that were euglycemic (normoglycemic). Each dog had hyperglobulinemia due to a myeloma producing monoclonal IgA. Binding of glucose to the IgA paraproteins was considered the reason for the increased fructosamine concentrations.[262]

D. Increases in glycated Hgb percentage occur in people with iron deficiency anemia and in some patients with hepatic disease and chronic renal failure.

E. Hemolytic or hemorrhagic anemia and RBC transfusions may mask or reduce increases in [glycated Hgb] (see the following section, Section IV).

IV. Decreased [fructosamine] and decreased glycated hemoglobin percentage or concentration (Table 14.7)

A. Decreased [fructosamine]

1. Hypoglycemia (persistent): Persistent hypoglycemia caused by an inappropriate release of insulin (e.g., insulinoma) can lead to a decreased serum [fructosamine].[164,263,264]
2. Hypoproteinemia or hypoalbuminemia
   a. Normoglycemic dogs with hypoalbuminemia had decreased fructosamine concentrations.[261,265]
   b. About 67 % of normoglycemic cats with hypoproteinemia had decreased serum [fructosamine].[261] The [fructosamine] was better correlated with [total protein] than with [albumin].
3. Cats with hyperthyroidism have significantly lower serum fructosamine concentrations than healthy cats;[266,267] similar findings are found in people with thyrotoxicosis.[268] The altered protein metabolism and thus altered [fructosamine] complicates the interpretation of [fructosamine] in cats that have both hyperthyroidism and DM, because their fructosamine concentrations may be within the RI.[119]
4. Decreased [fructosamine] has been associated with azotemia and hyperlipidemia in normoglycemic dogs but not cats.[261] However, the report did not describe the underlying disorders or whether the azotemic or hyperlipidemic dogs had dysproteinemias.

B. Decreased glycated Hgb percentage or concentration

1. Hypoglycemia (persistent): Persistent hypoglycemia caused by an inappropriate release of insulin (e.g., insulinoma) can lead to a decreased glycated Hgb percentage or concentration.[248,249]
2. Anemia: If [RBC] is stable, then the degree of glycated Hgb formation primarily depends on blood [glucose] during the life span of the RBCs.
   a. If there is an acute anemia (e.g., because of hemorrhage or hemolysis) and a subsequent regenerative response, then the glycated Hgb concentration and percentage are expected to be reduced because young RBCs have less glycated Hgb and blood [Hgb] is decreased. These decreases are also expected with decreased RBC survival that is adequately compensated (no anemia) by increased erythropoiesis.

(1) Decreased glycated Hgb percentages have been reported in dogs with regenerative and, to a lesser extent, nonregenerative anemias, correlating to some degree with RBC indices supportive of increased RBC turnover.[249,250,269]

(2) However, increased glycated Hgb percentages have been reported in dogs with nonregenerative and regenerative anemias caused by a variety of conditions when measured by turbidimetric immunoinhibition, a method that correlated poorly with [fructosamine] in one study.[270,271] Conflicting findings may relate to the different analytical methods (analytical accuracy) or to the dogs' conditions and preanalytical factors.

b. If there is anemia of any origin and the animal has not been hyperglycemic, then the [glycated Hgb] (not percentage) will be decreased because the blood [Hgb] is decreased.[249]

c. Increased glycated Hgb percentages have been repeatedly reported in people with iron-deficiency anemia;[272] RBC oxidative stress may contribute.

V. Correction of the [fructosamine] for abnormal protein concentrations

A. Because dysproteinemias affect [fructosamine] independently of blood [glucose], some authors have recommended correction formulas to adjust for the dysproteinemia (Eq. 14.2). The formulas suggest the relationships are completely linear and depend only on albumin or total protein concentrations, but the degree of glycation per mole of albumin depends also on the half-lives of the proteins. At a lower [albumin], the catabolism of albumin is decreased, so its half-life is increased. This results in greater glycation per mole of albumin, thus somewhat counteracting the decrease in fructosamine caused by hypoalbuminemia.[273] Consequently, the correlation between albumin and fructosamine concentrations is poor to moderate.

$$\text{Corrected canine [fructosamine]} = [\text{fructosamine}]_{\text{measured}} \times \frac{[\text{albumin}]_{\text{healthy dogs}}}{[\text{albumin}]_{\text{measured}}} \quad (\textbf{14.2a})$$

$$\text{Example: corrected canine [fructosamine]} = 180\ \mu\text{mol/L} \times \frac{3.3\ \text{g/dL}}{2.0\ \text{g/dL}} = 297\ \mu\text{mol/L}$$

$$\text{Corrected feline [fructosamine]} = [\text{fructosamine}]_{\text{measured}} \times \frac{[\text{total protein}]_{\text{healthy cats}}}{[\text{total protein}]_{\text{measured}}} \quad (\textbf{14.2b})$$

Note: The midpoints of appropriate RIs are used for albumin and total protein concentrations of healthy dogs and cats.

B. A canine correction formula using serum [albumin] was recommended because of a greater correlation coefficient (r) for [fructosamine] versus serum [albumin] ($r = 0.79$) than versus [total protein] ($r = 0.54$).[261] However, in one study involving diabetic and nondiabetic dogs, use of the corrected concentrations did not substantially increase the diagnostic sensitivity or diagnostic specificity of the [fructosamine] (nitroblue tetrazolium assay) for DM.[260]

C. A feline correction formula using serum [total protein] was recommended because of a greater correlation coefficient for [fructosamine] with serum [total protein] ($r = 0.68$) than with [albumin].[261] Because there was very poor correlation with serum [albumin], the authors suggested that a greater proportion of the glycated proteins were globulins.

IMMUNOREACTIVE INSULIN (IRI) CONCENTRATION

I. Physiologic processes
   A. Insulin is a polypeptide hormone ($M_r \cong 6000$) with 51 amino acids in two chains (A and B) linked by disulfide bridges. Mammalian insulins are quite homologous. The amino acid sequence of human insulin differs from the sequences of feline, bovine, equine, canine, and porcine insulins by four, three, two, one, and one amino acid(s), respectively.[274,275] Canine and porcine insulin sequences are identical, and the canine and bovine sequences differ by two amino acids. Feline insulin differs from porcine and bovine insulins by three and one amino acid(s), respectively. Canine and feline insulins differ from each other by three amino acids, as do equine and bovine insulins. Equine and porcine insulins differ by one amino acid.
   B. The major physiologic stimulus for insulin secretion is an increased concentration of glucose, but insulin secretion is promoted by increased concentrations of other nutrients including amino acids (glutamine, leucine, arginine), and by several hormones, including glucagon, glucagon-like peptide-1, growth hormone, gastric inhibitory peptide, gastrin, secretin, pancreozymin, gastrointestinal polypeptide, and β-adrenergic hormones.
   C. Inhibitors of insulin secretion include somatostatin, α_2-adrenergic agonists, β-adrenergic antagonists, and IGF-1.[156,276]
   D. Preproinsulin is made by ribosomes of pancreatic β cells and is quickly cleaved to proinsulin that is stored in secretory granules of the Golgi complex. Insulin is formed after cleavage enzymes (some of which are Ca^{2+} regulated) break peptide bonds to form insulin, C-peptide, and split peptides. The [glucose] regulates synthesis of proinsulin and one of the cleavage enzymes. Insulin and C-peptide secretions are equimolar; small amounts of proinsulin and split peptides are also released.
   E. The major actions of insulin are illustrated in Figure 14.1 (glucose related) and listed in Table 14.8 (glucose and lipid related).

II. Analytical concepts
   A. Terms and units
      1. *Immunoreactive insulin* is the preferred term for immunoassay measurements of serum or plasma insulin for two reasons: (i) measurements may include proinsulin, and (ii) measurements of insulin are in immunoreactive units, not biological activity units of injectable insulin.
      2. Units: μIU/mL (μU/mL) = mIU/L; μIU/mL × 6.00 = pmol/L, though other conversion factors based on other/older insulin products or imprecise calculations are often used;[279] μg/L × 172.2 = pmol/L (SI unit)[13]
   B. Assays
      1. Measurements of IRI are made using immunoassays (radioimmunoassays, chemiluminescent assays, or ELISAs) and therefore anti-insulin antibody reagents.[280,281]
      2. The antibody in commercial assays may have been developed to react with porcine or human insulin. There is sufficient cross-immunoreactivity (as much as 100 %) that commercial assays have been validated for canine insulin. Three of five commercial radioimmunoassays were considered acceptable for feline insulin.[282] An assay's antibody immunoreactivity with insulin, proinsulin, and split products varies with the antibody and the species. Cross-reactivity with synthetic insulin has been reported and is dependent on the antibodies and product.

Table 14.8. Major actions of GH, IGF-1, and insulin on glucose and lipids[277,278]

Target	IGF-1	GH	Insulin
Plasma	↓ [glucose][a]	↑ [glucose]	↓ [glucose]
Hypothalamus	↑ somatostatin synthesis ↓ GH secretion	—	—
Pancreas	↓ insulin secretion[a]	—	—
Skeletal muscle	↑ glucose uptake ↑ FA uptake and β-oxidation	↑ LPL activity[b]	↑ glucose uptake ↓ lipolysis ↑ glycogenesis
Kidneys	↓ gluconeogenesis	↑ gluconeogenesis	↓ gluconeogenesis
Liver	—	↑ IGF-1 synthesis ↑ gluconeogenesis ↑ glycogenolysis ↓ insulin activity ↑ β-oxidation of FA	↓ glycogenolysis ↑ glycogenesis ↑ glycolysis ↑ β-oxidation of FA[c] ↑ lipogenesis ↓ lipolysis ↓ gluconeogenesis ↑ IGF-1 synthesis ↓ IGFBP-1 synthesis
Adipose tissue	—	↓ insulin activity ↓ glucose uptake ↓ LPL activity[b] ↑ lipolysis[b] ↑ FA release	↑ glucose uptake ↑ lipogenesis ↓ lipolysis

[a] At high IGF-1 concentrations
[b] See Growth Hormone (GH) Concentration, Section I.
[c] Via catecholamines
Note: Indirect effects of IGF-1 on hepatocytes and adipocytes are not shown.

3. Wide ranges of [IRI] have been reported for canine, feline, and human samples assessed with commercial insulin assays. The variation is multifactorial and only partially due to differences in standard or calibrator solutions. Because of this variation:
   a. A patient's [IRI] should be compared against RIs established for the assay used to measure the patient's [IRI], and the assay should have been validated for the species being tested. Diagnostic decision limits for [IRI] or IRI:G ratios need to be established for each validated insulin (and glucose) assay.
   b. Laboratories offering quantitation of [IRI] should thoroughly evaluate their insulin assays for performance characteristics with varying lots of reagents.
4. Human assays have been used to measure [IRI] in llamas and alpacas.[283,284] Compared to other domestic mammals, these species release less insulin in response to glucose, and clear plasma glucose more slowly.

C. Samples
1. An [IRI] may be determined in serum or heparinized plasma. EDTA-plasma has been used as well, but equine [IRI] results generated by a chemiluminescent immunoassay were markedly decreased in EDTA-plasma compared to serum and heparin-plasma.[285]

2. Unless hypoglycemic (e.g., assessing for an insulinoma), dogs and cats should be fasted prior to sample collection because glucose, amino acids, and several gastric, intestinal, and pancreatic hormones influence insulin secretion. Grain consumption by horses may increase [IRI] for at least 5 h,[286] and the IRI:G ratio trends up with complete fasting, which by 6 h and clearly by 12 h attenuated the decrease in [glucose] induced by insulin administration.[287] Therefore, a recommendation for basal insulin assessment is to collect samples from nonfasted horses, but not within 5 h of feeding grain.[288] If this is not done, food intake should be considered when interpreting results.
3. Canine [IRI] was stable for 30 d in six serum samples stored at –20 °C and in most samples for 8 d at 4–8 °C; it was stable for < 3 d at room temperature (20 °C).[280] Equine [IRI] was stable for 8 d when whole blood collected into heparin tubes or clot activator tubes was held at 4 °C prior to harvesting serum or plasma, but for only 3 d at 20 °C.[285] Similar stabilities are expected for other species.

III. Hyperinsulinemia
   A. The major reason for measuring [IRI] is to document the inappropriate release of insulin from β cells, i.e., too much insulin released for the animal's plasma or serum [glucose]. This has been done most often to diagnose insulinomas in hypoglycemic dogs, cats, or ferrets, or to assess for equine metabolic syndrome or insulin resistance related to pituitary adenomas in normo- or hyperglycemic horses with PPID.
   B. Disorders and pathogeneses (Table 14.9)
      1. Increased insulin production and release
         a. Functional pancreatic β-cell neoplasm (insulinoma): Neoplastic β cells may consistently or sporadically produce insulin, which causes hypoglycemia because of enhanced glycolysis, reduced gluconeogenesis, and increased glucose uptake by myocytes and adipocytes.[164] It is important to interpret measured [insulin] in the context of a concurrent [glucose]; an insulin concentration within its RI when there is concurrent hypoglycemia is evidence of inappropriate insulin release (see Immunoreactive Insulin (IRI) Concentration, Section V).
         b. Hyperinsulinemia may also occur in other hypoglycemic disorders, including noninsulinoma pancreatogenous hypoglycemia (acquired nesidioblastosis), various hyperinsulinemic hypoglycemic disorders including congenital hyperinsulinism,[169] and xylitol toxicosis (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section IV.C.1.a).
         c. Hyperglycemia not caused by decreased insulin production

Table 14.9. Disorders and conditions that cause hyperinsulinemia

Increased insulin production and release
Functional pancreatic β-cell neoplasm (insulinoma)
Noninsulinoma pancreatogenous hypoglycemia (acquired nesidioblastosis)
Idiopathic hyperinsulinemic hypoglycemia
Xylitol toxicosis
Hyperglycemic disorders not caused by decreased insulin production (see text)
Anti-insulin antibodies (analytical error)

(1) Hyperinsulinemia is expected with physiologic hyperglycemia because hyperglycemia stimulates the production and release of insulin. Consequently, increases in [IRI] occur secondary to epinephrine or cortisol release in stressed or painful horses, including those with laminitis; insulin testing in such horses should be done after clinical laminitis and pain have subsided.

(2) Hyperinsulinemia is expected with pathologic and pharmacologic hyperglycemias if insulin release from β cells is not defective. Also, a state of insulin resistance may initiate or augment a hyperglycemic state and concurrent hyperinsulinemia. The hyperglycemia and suppression of insulin production caused by xylazine administration to horses is followed ~1.5–3 h later by increased [insulin] and potentially hyperinsulinemia, particularly in horses with insulin dysregulation.[153]

2. Anti-insulin antibodies: The presence of anti-insulin antibodies may result in a falsely increased [IRI] when certain assays are used.[289,290] Anti-insulin antibodies may arise from spontaneous pathologic processes or insulin therapy. Their concentration in a serum or plasma sample can be measured with immunoassays.[291]

IV. Hypoinsulinemia

A. Documenting hypoinsulinemia could help in classifying or characterizing DM states, e.g., confirming type 1 DM, staging type 2 DM, or assessing insulin status in other diabetic states.

1. Measuring [IRI] in hyperglycemic dogs and cats is not routine; [IRI] is more commonly assessed when patients are hypoglycemic. The infrequent measurement of [IRI] in DM cases is probably due to many factors, such as cost, lack of standardized assays, and lack of diagnostic or prognostic criteria associated with variations in [IRI] in different types of DM.
2. A deficiency in insulin can be defined in two ways: (i) [IRI] is below an appropriate RI, and (ii) a relative deficiency in which the amount of insulin is insufficient to maintain adequate carbohydrate metabolism. Fasting plasma [IRI] has been used as a method of detecting insulin resistance in cats fitting the second definition.[292]

B. Disorders or conditions for which [IRI] is expected to decrease

1. Pathologic hypoinsulinemia
   a. Type 1 DM: Insulin production is decreased because of the destruction of β cells.
   b. Type 2 DM: Advanced stages of pancreatic amyloidosis or glucotoxicity involve β-cell damage, impaired secretory function, and thus decreased insulin production (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section III.B.2.b).
2. Physiologic hypoinsulinemia: Animals with a variety of hypoglycemic states (see Table 14.5) would be expected to have hypoinsulinemia if the hypoglycemic state was not caused by increased insulin secretion.
3. In some assays, the presence of anti-insulin antibodies can cause falsely decreased values.

V. Immunoreactive insulin-to-glucose (IRI:G) ratio

A. Because insulin production and release from β cells depend on plasma [glucose], an IRI:G ratio should indicate whether the measured [IRI] is appropriate for the degree of glucose stimulation. When used, the ratio has typically been calculated by using conventional units for both IRI and glucose concentrations (Eq. 14.3), but SI units may be used.

$$\text{IRI:G} = \frac{[\text{IRI}] \times 100}{[\text{glucose}]} \quad (\mathbf{14.3})$$

with [IRI] in μIU/mL and [glucose] in mg/dL; thus, the IRI:G ratio unit is μIU/mg.

B. The lack of analytical agreement among insulin (and glucose) assays requires that RIs for IRI:G ratios be established for each assay, which is a task that is not commonly accomplished.
C. Interpretation of the fasting IRI:G ratio
   1. Increased IRI:G ratio
      a. If associated with hypoglycemia, then insulin is contributing to the hypoglycemia.
      b. If associated with normoglycemia or hyperglycemia, then an increased IRI:G ratio may indicate insulin resistance. When this ratio was calculated from values measured in samples from fasted cats, it was shown to be a relatively reliable method for assessing insulin sensitivity compared to several other methods.[292]
   2. IRI:G ratio within the RI
      a. If associated with hypoglycemia, then a pathologic state other than hyperinsulinemia is causing the hypoglycemia.
      b. If associated with hyperglycemia, then a factor other than insulin deficiency is causing the hyperglycemia.
   3. Decreased IRI:G ratio: If associated with hyperglycemia, then an insulin deficiency is present that could be due to β-cell damage or glucose toxicosis.
D. The IRI:G ratio may be difficult to interpret for reasons other than the variations created by different assays.
   1. Substances other than glucose (e.g., GH, glucagon, epinephrine) influence insulin release from β cells.
   2. Much of the released insulin is removed from portal blood by hepatocytes and thus does not appear in routine blood samples.
E. Amended IRI:G ratio (not to be used) and the "< 30 mg/dL theory"
   1. In 1971, Turner and associates reported an "observation that the plasma insulin levels of normal subjects [humans] are near zero if the plasma glucose is 30 mg/dL or less"[293] and referenced an article by Turner, Oakley, and Nabarro that was in press. Based on the observation, they proposed that a [glucose] above 30 mg/dL would result in insulin entry into systemic blood. To evaluate [IRI] in hypoglycemic people, they modified the IRI:G ratio by subtracting 30 mg/dL from the measured [glucose]. If the modification was valid, there would be a direct relationship between insulin and glucose concentrations over 30 mg/dL, i.e., *x* μIU of insulin for every *y* mg of glucose > 30.
   2. In 1973, Turner, Oakley, and Nabarro reported changes in plasma [insulin] during ethanol-induced hypoglycemia in obese and nonobese people.[294]
      a. They did not mention their "< 30 mg/dL" theory or proposed amended IRI:G ratio of 1971, and their published data were not consistent with the theory. Insulin was not detected in several samples with a [glucose] of 40–65 mg/dL. There were only three samples with a [glucose] < 30 mg/dL; insulin values in those samples ranged from 0–2.0 μIU/mL.
      b. They did write, "The fall in plasma insulin was a function of the fall, rather than of the absolute values of the plasma glucose."
      c. Another variable not considered was the difference between insulin concentrations in portal and routine blood samples. Because much of the secreted insulin is removed from portal blood by hepatocytes, assessment of insulin secretion stimulated by glucose is better evaluated by measuring portal blood concentrations, which is a technique generally limited to experimental investigations.

3. Many publications have included the use of the amended IRI:G ratio to evaluate [IRI] and [glucose] in domestic and nondomestic animals. There have also been several attempts to squelch the use of the amended IRI:G ratio, but it seems to have a life of its own.[295–299] The amended IRI:G ratio should not be used.

IMMUNOREACTIVE GLUCAGON (IRG) CONCENTRATION

I. Physiologic processes
   A. *Pancreatic glucagon* is a 29-amino-acid polypeptide hormone that is secreted by the α cells of the pancreas and cleared by liver, kidneys, and likely endovascular proteolysis. It forms from proglucagon, a 160-amino-acid peptide in pancreatic α cells and intestinal L cells, the latter being enteroendocrine epithelial cells named for their large (L) granules.[300,301] In addition to glucagon, pancreatic α cells secrete a proglucagon fragment (amino acids 1–61) that has the same carboxyl terminus as glucagon (this is relevant to immunoassay detection).
      1. The major role of glucagon is to maintain blood [glucose] during potentially hypoglycemic physiologic states of fasting, exercise, and stress. It does this by promoting hepatic glycogenolysis for rapid glucose availability and gluconeogenesis for more sustained glucose generation, as illustrated in Figure 14.1. Glucagon also appears to promote cellular uptake of amino acids, lipolysis, hepatic FA oxidation (generates energy for gluconeogenesis), and ketogenesis.
      2. Stimuli for pancreatic glucagon secretion include hypoglycemia (major stimulus), postprandial increases in amino acids, hypercortisolemia, the autonomic nervous system, epinephrine, and probably hypoinsulinemia. In healthy neonatal foals, fasting did not increase [IRG], but [IRG] increased within 30 min of nursing after a 3-h fast, as did [IRI] and [glucose].[302]
      3. Glucagon's impact on plasma [glucose] depends on its balance with insulin and other metabolic mediators, so [glucose] may not reflect [IRG], and vice versa.
   B. Proglucagon in intestinal L cells may generate small amounts of glucagon, but posttranslational cleavage primarily generates glucagon-like peptides 1 and 2 (GLP-1, GLP-2), glicentin, and oxyntomodulin, all of which are enteropeptides that influence or regulate digestive and metabolic processes.
      1. *GLP-1* is released from L cells after feeding. It enhances nutrient-stimulated release of insulin, inhibits glucagon release, and reduces postprandial hyperglycemia. Synthetic analogs of GLP-1 have been used for treatment of DM in people and cats.[303]
      2. Prior to the identification of GLP-1, the hormone was referred to as gut glucagon, which cross-reacted with glucagon in some immunoassays.

II. Analytical concepts
   A. Terms and units
      1. The terms *immunoreactive glucagon (IRG)* or *glucagon-like immunoreactivity* can be used to describe what is measured by glucagon immunoassays; in addition to glucagon, this may include glucagon-like peptides, depending on antibody specificity.
         a. Human carboxy-terminal immunoassays that use monoclonal antibodies targeting the carboxyl terminus of glucagon also detect the 61-amino-acid proglucagon fragment released from the pancreas. Amino-terminal immunoassays use monoclonal antibodies that react with oxyntomodulin (intestinal) in addition to glucagon.
         b. One group of investigators described validation studies using an anti-(bovine glucagon) antibody to assess [glucagon] in dogs, cats, sheep, cows, and horses. In the

fasting state, about 30 % of the IRG was pancreatic glucagon. Their antibody cross-reacted with larger molecular forms that had glucagon-like immunoreactivity.[304]

2. Unit conversion: pg/mL = ng/L (SI unit);[13] ng/mL × 0.2871 = pmol/L

B. Assays
   1. Because of nonspecific interactions of anti-glucagon antibodies with plasma proteins, some investigators recommend pretreatment to extract interfering proteins. However, ethanol extraction has recovered only about 70 % of sample glucagon (~40–90 %), making results inaccurate for reasons other than plasma protein interference.[300]
   2. For many years, the gold standard assay for canine IRG was a radioimmunoassay with Unger's 30K antibody, an antibody targeting the C terminus of glucagon.[305] More recently, a commercial monoclonal antibody-based human sandwich ELISA has been used for dogs,[306] but published peer-reviewed validation studies for use in dogs are lacking. A human double-antibody RIA has been used to measure IRG in feline EDTA-plasma, but very limited method validation data have been published to support use in cats.[307] Different glucagon immunoassays continue to generate different results because of differences in sample preparation, antibody specificities, reference standards, assay matrix, and effects of interfering factors in plasma or buffers.[308,309]
   3. Liquid chromatography with tandem mass spectrometry can be used to measure [glucagon] without concern about antibody reactivity, but other factors that can affect the accuracy of results must be considered.[300,308]
   4. Assays for human GLP-1 have also been developed and employed in veterinary medicine but are beyond the scope of this book.[306,310]

C. Samples
   1. Plasma from EDTA-anticoagulated blood is most commonly recommended and used, but heparin-plasma (lithium or sodium salts)[128,308] and serum[311,312] have also been used.
   2. Sample recommendations:[300,308,311] Glucagon and IRG instability necessitates careful sample handling. There is evidence that enzymatic degradation and therefore stability of glucagon varies among species,[313] so an abundance of caution is reasonable in the absence of species-specific studies.
      a. For plasma samples, whole blood should be immersed in an ice bath immediately after collection and sample inversion. It is best to promptly separate plasma from cells in a 4–5 °C centrifuge, and with forces appropriate for removing all cell types, including platelets, from the plasma. When plasma was harvested from whole blood stored for 24 h at refrigerator temperature, there were mild to marked decreases in [IRG].
      b. EDTA-plasma or serum samples are best refrigerated (2–8 °C) for no longer than 3–6 h prior to analysis, or concentrations will be falsely decreased; samples can remain at room temperature for up to 1 h.
      c. Recovery of spiked synthetic glucagon in samples stored for as little as 3 h at –20 °C was approximately 50 %, but an assay manufacturer has reported stability of endogenous glucagon in EDTA-plasma for up to and not exceeding 12 mo at –20 °C (provided data suggest some false increases at 12 mo).[311] Long-term storage at –80 °C is safest, reportedly maintaining [glucagon] stability in EDTA-plasma and heparin-plasma for 9 mo.[308]
      d. Commercial sample tubes containing an antiprotease cocktail (not addition of aprotinin) have been recommended when assessing for decreases in [glucagon],[311] but they do not appear to be necessary for fasted samples that are processed immediately and promptly assayed or frozen as described. Antiproteases may be helpful with deviations from recommended sample processing, but patient samples and RI samples should be processed the same way.

III. Hyperglucagonemia
- A. Because glucagon is a hormone involved in carbohydrate and lipid metabolism, there are many reports of IRG concentrations in various physiologic and pathologic states for which glucagon testing is not clinically indicated. Those results are not considered here, partly because interpretation of [IRG] results in domestic mammals is limited by incomplete characterization and validation of assays.
- B. Neoplasms: Increased plasma [IRG] has been reported in dogs with glucagon-producing pancreatic neoplasms (e.g., glucagonoma), with or without superficial necrolytic dermatitis.[314,315] Other dogs have had superficial necrolytic dermatitis, glucagon-positive pancreatic neoplasms by immunohistochemistry, and hyperglycemia, but plasma glucagon concentrations were not measured.[106]
- C. The [IRG] reported for dogs with evidence of aminoaciduric hypoaminoacidemic hepatopathy syndrome, which, as with glucagonomas, can occur with superficial necrolytic dermatitis, have been low, WRI, or increased.[306,314,316–318] In the most recent evaluation with application of a modern sandwich ELISA for human glucagon, affected dogs had decreased fasting and postprandial concentrations of IRG compared to healthy dogs.[306] However, the study was small, the duration of stability for [IRG] in canine heparin-plasma samples at –20 °C is not clear, and manufacturer-independent validation of the assay for canine samples has not been reported.

GROWTH HORMONE (GH) CONCENTRATION

I. Physiologic processes
- A. GH (somatotropin) is a heterogeneous polypeptide hormone produced in multiple forms by somatotroph cells of anterior pituitary glands; it is also produced by canine mammary epithelial cells when stimulated by progestins.[319,320] Its major actions involve growth, cell reproduction, and cell regeneration. GH secretion is stimulated by GH-releasing hormone (somatocrinin) and inhibited by GH-inhibiting hormone (somatostatin), increased [IGF-1], and increased [GH] (which promotes IGF-1 secretion). Major actions of insulin, IGF-1, and GH are listed in Table 14.8 and shown in Figure 14.1.
- B. GH promotes insulin resistance and hyperglycemia through increased gluconeogenesis, increased glycogenolysis, and decreased uptake of glucose by adipocytes. A lower [GH] indirectly enhances hepatic insulin activity by reducing antagonism of insulin.
- C. Increased [GH] decreases LPL activity in adipose tissue and therefore decreases lipid storage.[321,322] It increases adipose tissue lipolysis by hormone-sensitive lipase, thus promoting increased plasma [FA].[323] In contrast, increased [GH] increases LPL activity in skeletal muscle, promoting cellular uptake of FAs and accumulation of lipid intermediates that impede insulin signaling pathways.[322,324]

II. Analytical concepts
- A. Sample: Serum is the preferred sample. Feline GH concentrations were stable up to 24 h at room temperature; serum should be frozen (–20 °C or –80 °C) for longer storage. In matched samples, GH concentrations in EDTA plasma were greater than in serum.[325]
- B. Assay availability is limited. GH is often assessed indirectly by assessment of IGF-1, which has a longer plasma half-life than GH and is produced in response to increased [GH]. There is sufficient cross-immunoreactivity of GH assays for ovine, canine, or human GH that they can be used to measure feline and canine [GH] by immunoassays

Table 14.10. Disorders and conditions that cause increased [GH]
Hypersomatotropism due to a somatotroph adenoma*
GH-producing mammary neoplasms and ovarian cysts in dogs
Primary hypothyroidism in dogs
Diestrus and pregnancy in dogs*

* A relatively common disorder, condition, or mechanism

including radioimmunoassays, ELISAs, and chemiluminescent assays.[102,325–327] However, differing calibration standards and various specificities of assay antibodies for the various molecular forms of GH likely affect results for domestic mammals as they do in human medicine.[328] RIs need to be established for each assay because of lack of standardization.

III. Increased GH concentration (hypersomatotropinemia) (Table 14.10)

A. Hypersomatotropism due to a somatotroph adenoma

1. There are two major clinical manifestations of excessive GH activity (hypersomatotropism): acromegaly and DM. Features of acromegaly (*akron* – tip, extremity; *megas* – great) include enlargement and thickening of head and feet (due to increased bone growth) and organomegaly (heart, kidneys). The diabetic state is characterized by insulin resistance and increased [IGF-1]. Animals with acromegaly have at least prediabetic glucose intolerance. Animals with a somatotroph adenoma and DM may not have clinical acromegaly. Because of sporadic GH secretion, a single sample may not yield an increased [GH], but nearly all cases have an increased [IGF-1] created by GH-stimulated production of IGF-1 by hepatocytes.
2. DM is common in feline hypersomatotropism. Increased [IGF-1] is used to confirm the pathologic state (see Insulin-Like Growth Factor-1 (IGF-1) Concentration, Section III).[90,91,93,110]

B. GH-producing mammary neoplasms and ovarian cysts in dogs: Canine acromegaly has been caused by GH-producing mammary neoplasms[103,329] and ovarian cysts.[329] Swedish elkhounds with mammary masses had greater [GH] than those without mammary masses.[102]

C. Primary hypothyroidism in dogs: Increased [GH] and [IGF-1] have been found in dogs with primary hypothyroidism.[330,331] Thyrotropin-releasing hormone given to hypothyroid dogs caused increased plasma GH concentrations and reduced release of thyrotropin, whereas it did not increase [GH] in dogs with hypothyroxemia caused by nonthyroidal illness (TSH within RI).[332] The increased [GH] is due to transdifferentiation of somatotropic pituitary cells (i.e., thyrotropin-secreting cells develop the ability to secrete GH) and thyrotroph hyperplasia.[330,331]

D. Diestrus and pregnancy in dogs: Compared to anestrus, [GH] is increased during the luteal phase of diestrus in healthy cycling bitches.[333] The increase in progesterone promotes production of GH by the canine mammary gland, and GH further promotes mammary development and insulin resistance. Signs of acromegaly develop in some bitches during such periods of high progesterone production.[57] There is a relatively high incidence of Swedish elkhounds developing progesterone-related DM during diestrus and pregnancy, times when there is progesterone-induced secretion of GH by mammary glands, and insulin resistance in older pregnant nondiabetic Swedish elkhounds was associated with increased [GH].[102] Administration of progestins to ovariohysterectomized dogs also increases plasma [GH].

Table 14.11. Disorders and conditions that cause decreased [GH]
Congenital hyposomatotropism (pituitary dwarfism)
Acquired hyposomatotropism

IV. Decreased GH concentrations (hyposomatotropinemia) (Table 14.11)
  A. Congenital hyposomatotropism (pituitary dwarfism)
    1. Hyposomatotropism is the clinical manifestation of decreased [GH]. When it results from a congenital defect, it is called congenital hyposomatotropism or pituitary dwarfism. Besides decreased [GH], there may be decreased production of other pituitary hormones (e.g., thyrotropin, ACTH), which results in other endocrinopathies.[334]
    2. Feline congenital hyposomatotropism has been described.[335,336]
    3. Canine congenital hyposomatotropism is an inherited disorder in German shepherd, spitz, miniature pinscher, and Karelian bear-dog breeds.[337–339] Affected Saarloos and Czechoslovakian wolfdogs, both of wolf and German shepherd breeding, have the same mutation as German shepherds. A different mutation was identified in a hyposomatotrophic Chihuahua.[201]
  B. Acquired hyposomatotropism may result from a variety of pathologic or iatrogenic states that cause decreased pituitary function. An acquired case occurred in a young cat after being bitten in the head by a dog.[200] The cat had decreased [GH], subsequently decreased [IGF-1], and hypoglycemia.

INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) CONCENTRATION

I. Physiologic processes[278]
  A. IGF-1 (somatomedin C) is a polypeptide produced by many cells for local effects, but plasma IGF-1 is produced primarily by hepatocytes secondary to stimulation by GH, insulin, and the intake of calories and protein. The major actions of IGF-1 are via free IGF-1, the availability of which is regulated by plasma concentrations of IGF-1 binding proteins (IGFBPs). IGF-1 binding to IGFBPs and a third protein called acid labile subunit prolongs the plasma half-life of IGF-1 from minutes to hours. There are at least six IGFBPs; in people and cats, IGFBP3 carries most plasma IGF-1.
  B. IGF-1 shares molecular homology with IGF-2 and proinsulin, and consequently has some insulin activity, but IGF-1 supports structural growth while insulin mostly regulates ingested nutrients. Human IGF-1 is a polypeptide of 70 amino acids.
  C. IGF-1 is the major mediator through which GH exerts its effects on growth; genes for IGF-1, its receptor, and the GH receptor are important in determining dog size.[340] IGF-1 works in concert with GH and insulin to regulate glucose and lipid metabolism, making it unclear how much of the glucose-lowering effect of IGF-1 is direct and how much is secondary to suppression of GH (Table 14.8 and Figure 14.1).
    1. A major action of IGF-1 is to increase somatostatin production by the hypothalamus and by delta cells in pancreatic islets, stomach, and duodenum. Somatostatin reaching the pituitary gland via hypothalamic axons reduces GH synthesis and secretion and therefore promotes insulin actions in the liver. IGF-1 also promotes myocyte uptake of glucose (about 4–7 % of insulin activity) and FAs.

2. IGF-1 initiates cellular responses by binding to the almost ubiquitously expressed IGF-1 receptor (IGF-1R), an insulin receptor (IR), or to a hybrid IGF-1R/IR receptor. IGF-1 has much greater affinity (about 1000×) for IGF-1R than for IR. Expression of IGF-1R on hepatocytes is limited or absent in health, so physiologic effects of IGF-1 on hepatocyte metabolism are thought to be indirect. Hybrid IGF-1R/IR receptors are upregulated in muscle and adipose tissue during insulin resistance. The molar concentration of IGF-1 is much greater than the molar concentration of insulin; however, insulin is a free molecule whereas most plasma IGF-1 is bound to IGFBPs and therefore not active.
3. In the adult mammal, physiologic [IGF-1] has little direct effect on adipose tissue. However, high [IGF-1] does inhibit insulin secretion and thus lower [insulin] and promote lipolysis and mobilization of FAs.
4. With caloric restriction, mammalian hepatocytes secrete less IGF-1 and are refractory to GH stimulation. After a meal, the processes are reversed.

D. Serum [IGF-1] can vary severalfold with body size,[180,341] thus limiting the value of general population-based RIs, particularly for dogs because dog breeds vary tremendously in size. The effect has been reported in cats,[342] but weight gain alone did not increase [IGF-1], and obesity may decrease it.[343]

II. Analytical concepts

A. Sample: Serum and EDTA-plasma are typically used for IGF-1 immunoassays. Release of IGF-1 from platelets during clotting may contribute to mildly greater results when using serum, a difference that could be magnified in individuals with thrombocytosis. EDTA is better than heparin for inhibiting platelets and coagulation.

B. Stability: In one study, a statistically significant increase in serum [IGF-1] was present after 24 h of storage at room temperature, and clinically significant increases were present by 48 h; plasma [IGF-1] was stable for 24 h with room-temperature storage, and mild increases at 48 h were not statistically significant.[344]

C. Accurate measurement of [IGF-1] with immunoassays has been difficult because of the interference caused by IGFBPs binding to sample or standard IGF-1 and reliance on human (rather than species-specific) IGF-1 standard solutions, thus preventing assessment of accuracy in domestic species. Commercial human assays have been designed to measure [total IGF-1] after removing IGFBPs by a variety of methods; these include acid chromatography, alcohol or acid-alcohol extractions ± chemical displacement of residual IGFBPs, glycyl-glycine extraction, and/or use of excess IGF-2 as a blocking agent. Some of these assays have been assessed for measuring canine, feline, and equine [IGF-1].[102,342,344,345] Studies comparing results of the different methods, including different standard solutions, extraction methods, antibodies, and patient populations, have revealed significant analytical differences.[345–347]

D. Parallel reaction monitoring high-resolution mass spectrometry has been used to measure feline and canine serum concentrations of IGF-1 concurrently with IGF-2 and serum IGFBPs.[180,348]

III. Increased IGF-1 concentrations (Table 14.12)

A. Hypersomatotropism

1. Cats
   a. A cat with a somatotroph and corticotroph pituitary adenoma had increased [IGF-1] and [GH] with clinical features of DM and hyperadrenocorticism.[110]

Table 14.12. Disorders and conditions that cause increased [IGF-1]
Hypersomatotropism*
Anestrus bitches with experimentally induced hypothyroidism
Primary hypothyroidism*
Heart failure due to mitral regurgitation
Diet-induced obesity*

* A relatively common disorder, condition, or mechanism

b. In serum samples from 1221 cats with DM, 319 (26 %) had [IGF-1] > 1000 ng/mL, a medical decision limit considered supportive of hypersomatotropism with the assay that was used; most of these cats did not have physical features of acromegaly. Of the 63 diabetic cats assessed by computed tomography of the brain, 56 had pituitary neoplasms (somatroph adenomas). IGF-1 concentrations were measured by an RIA after acid-ethanol extraction.[91]

c. In a different study using a different IGF-1 assay, the [IGF-1] in cats with DM was shown to increase by 2–4 wk after initiation of insulin treatment, and it became > 1000 ng/mL in some cats that lacked any evidence of acromegaly.[348] The effect of treatment on [IGF-1] in cats with DM should be recognized when considering the diagnosis of feline hypersomatotropism.

2. Dogs: Rare canine cases of acromegaly due to hypersomatotropism have been reported. Insulin resistance and increased concentrations of GH and IGF-1 accompanied acromegaly in one dog.[95] Another dog had typical features of DM.[94]

B. Increased IGF-1 concentrations have been reported in various canine disorders other than hypersomatotropism; variations in IGF-1 are found in these disorders because of variations in GH concentrations, insulin concentrations, or other factors affecting regulation of glucose or lipid metabolism.

1. Anestrus bitches with experimentally induced hypothyroidism had increased [IGF-1], increased [GH], and increased insulin response to hyperglycemia. The increased [IGF-1] was considered an adaptive change related to insulin resistance.[122]
2. Dogs with primary hypothyroidism frequently have increased [IGF-1] due to increased GH stimulation (see Growth Hormone (GH) Concentration, Section III.C).[331]
3. In dogs with naturally occurring mitral regurgitation, IGF-1 concentrations after development of heart failure were greater than those during early development of heart failure and after successful treatment of heart failure.[349]
4. In dogs with diet-induced obesity, weight gain was positively correlated with increased [IGF-1] and negatively correlated with insulin sensitivity.[350,351]

IV. Decreased IGF-1 concentrations (Table 14.13)

A. Hyposomatotropism: Decreased [IGF-1] is an expected finding in animals with decreased [GH] because GH is the primary stimulant for IGF-1 production. Disorders due to decreased [GH] are described above.

B. Untreated DM: Decreased [IGF-1] occurs in some dogs and cats with DM, but concentrations may be increased after insulin therapy.

C. Restricted food intake: Decreased caloric intake reduces [IGF-1]. This has been shown experimentally in dogs via starvation[352] and restricted food intake.[353]

Table 14.13. Disorders and conditions that cause decreased [IGF-1]
Hyposomatotropism
Untreated diabetes mellitus
Restricted food intake
Congenital hypothyroidism
GM1 gangliosidosis
Hepatic disorders (documented in dogs)*

* A relatively common disorder, condition, or mechanism

D. Congenital hypothyroidism: [IGF-1] was decreased in a kitten with congenital hypothyroidism, and it increased during thyroxine therapy.[354]
E. GM1 gangliosidosis: Decreased IGF-1 concentrations were found in young cats with GM1 gangliosidosis; the neuronal dysfunctions of gangliosidosis may have reduced GH and consequently IGF-1 production.[355]
F. Hepatic disorders: Of 36 dogs with neoplastic and nonneoplastic hepatic disorders, 34 had lower IGF-1 concentrations than clinically healthy dogs. Of 20 dogs with nonhepatic disorders (mostly inflammatory), 10 had lower IGF-1 concentrations than clinically healthy dogs.[356] Two deerhounds with congenital portosystemic shunts had decreased IGF-1 concentrations,[357] and [IGF-1] has been documented to increase after surgical correction of portosystemic shunts.[358] Similar reports of feline hepatic diseases and IGF-1 concentrations were not found.
G. Endotoxemia: Experimental endotoxemia decreased [IGF-1] in dogs, and there is evidence of decreased IGF-1 production with other inflammatory states.[359,360]

INSULIN-LIKE GROWTH FACTOR-2 (IGF-2) CONCENTRATION

I. Physiologic processes
A. IGF-2 is a polypeptide sharing molecular homology with proinsulin and having insulin-like biological activity. It shares growth-promoting activity during in utero development. It has its own primary receptor that internalizes IGF-2 and reduces IGF-2 signaling, but it effects cell signaling by binding to IGF-1 receptors and insulin receptors; it also binds IGFBPs, which help regulate the amount of free (biologically active) and diffusible forms of IGF-2 as they do for IGF-1.
B. Via its binding to IGF-1 receptors and insulin receptors, IGF-2 can promote glucose uptake by myocytes, decrease glucose output by hepatocytes, decrease insulin secretion, and decrease glucagon secretion.[176] These actions are minor contributors to glucose homeostasis in physiologic conditions.
C. Hepatocytes might be the primary site of production, but many other cells are known to produce IGF-2. In contrast to IGF-1, IGF-2 secretion is largely independent of GH.

II. Analytical concepts
A. Sample: Serum and plasma have been used in immunoassays for [IGF-2].[177,361,362] Serum was also used in a targeted parallel reaction monitoring mass spectrometry method that concurrently analyzed for IGF-1, IGF-2 precursors (big IGF-2), and IGFBP3.[180] Stability studies for canine and feline samples were not found.

B. Immunoassays used to measure canine [IGF-2] include immunoradiometric assays after chromatographic separation[361] or after acid-alcohol extraction,[363] and ELISAs.[362]

III. Increased IGF-2 concentrations
   A. Because of its insulin-like activity and binding to insulin-receptors on target cells, increased IGF-2 activity may cause hypoglycemia. Hypoinsulinemia may also be present, thus excluding an insulinoma as a cause of hypoglycemia.
   B. Increased [IGF-2] may be the primary agent of paraneoplastic hypoglycemia associated with non–beta cell neoplasms including canine hepatocellular carcinoma,[177,363] canine mammary carcinoma,[178] canine gastric and splenic leiomyomas,[179,180] canine jejunal leiomyosarcoma,[177] and possibly equine renal carcinoma[181] (see Glucose Concentration in Serum, Plasma, or Whole Blood, Section IV.C.1.b). IGF-2 can mediate paraneoplastic hypoglycemia with or without an increased [IGF-2]; in some cases, the serum concentration of IGF-2 precursor molecules may be increased.[180]
   C. Increased [IGF-2] can be found in hypoglycemic dogs that have pancreatic islet cell neoplasms secreting IGF-2.[166,361]
   D. [IGF-2] may be increased in animals with inflammatory diseases. Dogs with generalized demodicosis had greater IGF-2 concentrations than healthy dogs; the dogs with demodicosis had slightly lower glucose concentrations than healthy dogs but the values were still within common RIs.[362]

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 15

EXOCRINE PANCREATIC AND INTESTINAL FUNCTIONS

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Table 15.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Exocrine pancreatic insufficiency
GFR	Glomerular filtration rate
HCO_3^-	Bicarbonate
PLI	Pancreatic lipase immunoreactivity
RBC	Red blood cell, erythrocyte
RI	Reference interval
RIA	Radioimmunoassay
SI	Système international d'unités (International System of Units)
TLI	Trypsin-like immunoreactivity
URL	Upper reference limit
WRI	Within reference interval
α_1-PI	α_1-Proteinase inhibitor

EXOCRINE PANCREATIC AND INTESTINAL ABSORPTIVE MALFUNCTIONS

Disorders of the exocrine pancreas or intestinal tract may alter body fluids and cause changes in routine laboratory test results. However, such changes are usually nonspecific; for example, neutrophilia, hypocalcemia, and increased alkaline phosphatase activity commonly occur with acute pancreatitis, but they also occur with many other pathologic conditions. Therefore, diagnosis of exocrine pancreatic or intestinal disease typically involves ancillary laboratory tests, the most useful of which are discussed in this chapter. These tests are used for assessment of the following disorders, which may occur together or independently.

I. *Exocrine pancreatic insufficiency* (EPI), recognized in dogs and cats, is a pathophysiologic state in which inadequate pancreatic secretions (enzyme rich or HCO_3^- rich) cause incomplete digestion of food (maldigestion). EPI may cause secondary intestinal abnormalities, such as increased mucosal maltase and sucrase activities, increased microvillar membrane proteins, and intestinal dysbiosis that may lead to more mucosal changes and malabsorption.
   A. Loss of acinar cells is extensive before there is clinical evidence of maldigestion, which is typically weight loss and malformed feces despite a good appetite.
   B. Pancreatic maldigestion may result from inadequate secretion of lipase, α-amylase, trypsinogen, chymotrypsinogen, carboxypeptidases, or combinations of these zymogens and enzymes.
   C. Two main pancreatic conditions are currently recognized as causes of EPI in dogs and cats.
      1. *Pancreatic acinar atrophy* (also known as juvenile pancreatic atrophy): A common cause of clinical EPI in dogs but rarely reported in cats, atrophy is thought to occur because of progressive immune-mediated damage to exocrine pancreatic cells (lymphocytic pancreatitis).
         a. In dogs, there is a complex mode of inheritance that may involve multiple genes.[1] Genetic risk and protective alleles have been identified in German shepherds and Pembroke Welsh corgis, including a risk allele identified in both breeds.[2,3]

b. Juvenile pancreatic atrophy has been reported in greyhounds, in which EPI and insulin-dependent diabetes mellitus develop concurrently.[4]

2. Chronic pancreatitis in dogs[5] and cats:[6] This disorder is typically idiopathic with recurring clinical bouts of pancreatitis that cause extensive destruction of acinar cells. If there is concurrent destruction of islet cells, diabetes mellitus may develop.

D. Pancreatic duct obstruction may impair passage of pancreatic enzymes into the intestine, thus causing maldigestion, but such an obstructive lesion would probably cause acute inflammation and presentation for an acute illness rather than for a maldigestive state with chronic weight loss or chronic diarrhea.

II. Pancreatitis

A. Even though the initiating factors for pancreatic inflammation are not thoroughly understood, it is known that pancreatic acinar cell damage is a major consequence of the inflammation.

B. Pancreatitis may be considered *acute* or *chronic*, the former occurring suddenly and the latter being persistent and associated with decreased pancreatic function and irreversible histologic changes such as fibrosis and acinar loss.[7] However, because the pancreas is not routinely evaluated histologically, it is often not possible to determine if pancreatitis with an acute clinical presentation has an underlying chronic component.[8]

C. In acute pancreatitis (from mild edematous to severe necrotizing or hemorrhagic), the release of cytoplasmic contents from damaged acinar cells can increase serum α-amylase or lipase activities (see Chapter 12, α-Amylase and Lipase), as well as concentrations of other molecules in serum, plasma, or urine (see following sections on TLI and PLI). Among domestic mammals, acute pancreatitis is most common in dogs. Affected dogs frequently have an acute onset of clinical signs such as vomiting and cranial abdominal pain.

D. Chronic pancreatitis may result from recurrent episodes of acute pancreatitis or from slowly progressive destruction of pancreatic acinar cells. When severe, the pancreas may not be able to secrete sufficient enzymes to digest food, and the animal may develop EPI. Endocrine pancreatic dysfunction may also arise. Chronic pancreatitis is considered to be more common than acute pancreatitis in cats, and chronic pancreatitis may be underreported in dogs.[9,10]

III. Intestinal malabsorption

A. Several small intestinal diseases may cause malabsorption of nutrients. The malabsorptive state could be localized (e.g., proximal small intestine or ileum) or diffuse; it could involve malabsorption of many nutrients (e.g., sugars, proteins, and fats) or be very specific (e.g., cobalamin). An affected animal is often presented because of weight loss or malformed feces. Acute enteric diseases that cause diarrhea for a few days probably cause a temporary malabsorptive state, but such disorders are not typically considered in discussions of malabsorptive disorders because there is not a concurrent malnourished state.

B. Intestinal diseases that lead to malabsorption occur in most animal species, but laboratory tests are used mostly to evaluate the disorders of dogs and occasionally of cats and horses. Specific diagnosis of suspected primary intestinal disease usually requires histologic examination of intestinal tissue. Examples of intestinal diseases that cause malabsorption include the following:

1. Inflammatory: lymphocytic enteritis, lymphocytic-plasmacytic enteritis, eosinophilic enteritis, granulomatous enteritis, histoplasmosis, pythiosis, giardiasis, and protothecosis

2. Neoplastic: lymphoma
3. Intestinal lymphangiectasia (inflammatory, neoplastic, or other cause)

TRYPSIN-LIKE IMMUNOREACTIVITY (TLI) CONCENTRATION IN DOGS, CATS, AND HORSES

I. Physiologic processes (Figure 15.1)

II. Analytical concepts
   A. Terms and units
      1. Immunologic assays detect multiple trypsin-like antigens, namely cationic trypsinogen, trypsin, and trypsin bound to protease inhibitors (probably α_1-antitrypsin), thus the name *trypsin-like immunoreactivity*. In healthy animals, nearly all TLI is from trypsinogen.

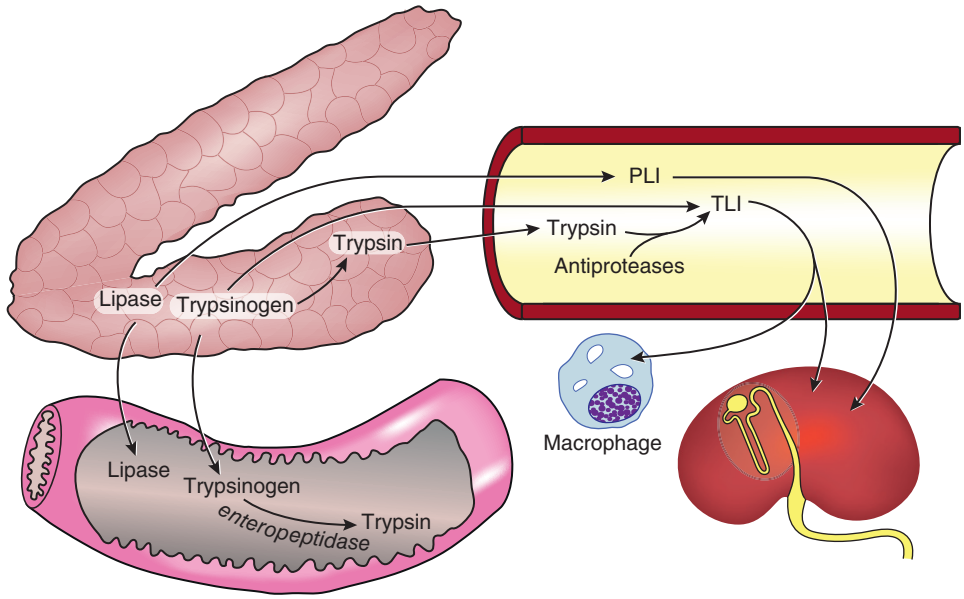


Figure 15.1. Physiologic processes that influence plasma or serum concentrations of TLI and PLI.

- TLI: Most trypsinogen is secreted in enzyme-rich pancreatic secretions into the intestine, where it is converted by intestinal enteropeptidase (EC 3.4.21.9) to trypsin, a potent digestive protease. In health, a small amount of trypsinogen escapes the pancreas and enters the blood, in which it can be measured as [TLI]. Also, small amounts of trypsin may be formed in the pancreas; this trypsin may enter the blood, bind to antiproteases, and contribute to [TLI]. Plasma trypsinogen and trypsin are cleared by the kidneys and by the mononuclear phagocyte system.
- PLI: Most pancreatic lipase is secreted in enzyme-rich pancreatic secretions into the intestine, where it catalyzes the lipolysis of dietary triglycerides. In health, a small amount of pancreatic lipase escapes the pancreas and enters the blood, in which it can be measured as [PLI]. The kidneys are involved in the removal of lipase from the plasma.

2. TLI is reportedly pancreas specific, and values were markedly decreased after pancreas removal from dogs; however, TLI was still measurable.[11] Trypsin is present in the human small intestine (Paneth cells), in bile epithelium, and in ovarian and hepatobiliary neoplasms.
3. Units: µg/L (SI units not found)

B. Assays
1. Canine, feline, and equine [TLI] is measured by species-specific immunoassays (RIA, chemiluminescent immunoassay, or ELISA). Trypsin's enzymatic activity is not measured because of the presence of trypsin inhibitors in serum.
2. RIs have differed substantially between ELISA and RIA methods,[12] indicating potential assay bias and the need to use appropriate RIs (see Reference Intervals, Chapter 1).

C. Sample: Serum is the preferred sample. Although laboratories offering TLI testing state that [TLI] is stable at room temperature for 3 d or 2 wk, and [TLI] in healthy dogs was reportedly unchanged when frozen for 5 yr,[13] published stability data were not found for samples from healthy or ill animals. Short-term storage at 4 °C and long-term storage at −20 °C have been recommended.

D. Published data regarding potential interference by hemolysis, hyperbilirubinemia, or lipemia were not found, but results from a laboratory that offers TLI testing and reduces lipemia by centrifugation prior to analysis showed no association between [TLI] and the presence of precentrifugation lipemia in a small study of postprandial samples from clinically healthy dogs.[14] In the same study, some individual changes in [TLI] were great enough to alter interpretation, but there was no consistent postprandial pattern of change.

III. Increased [TLI] (Table 15.2)

A. Increased release from pancreatic acinar cells
1. Acinar cell damage caused by pancreatitis[15,16]
a. Trypsinogen or trypsin released from damaged acinar cells enters blood, probably via lymph and peritoneal fluid.[17]
b. Dogs: A moderately to markedly increased serum [TLI] in a nonazotemic dog is supportive of pancreatitis, but pancreatitis may be present without an increased serum [TLI].
(1) In an experimental canine pancreatitis study, serum [TLI] tended to parallel serum α-amylase and lipase activities, i.e., all started to increase within 1 d, remained increased through day 5, and had returned to near baseline values at 2 wk. However, the peak in [TLI] occurred 1–2 d prior to the peak activities of α-amylase and lipase.[18]

Table 15.2. Disorders and conditions that cause increased [TLI] or [PLI] in dogs and cats

Increased release of trypsinogen (or trypsin) or lipase from pancreatic acinar cells
Acinar cell damage caused by pancreatitis, necrosis, or other disorders (see the text)*
Stimulated by food intake[a] or cholecystokinin and secretin administration (mild)
Decreased renal clearance ([TLI], possibly [PLI]) (see the text)
Cobalamin deficiency in cats ([TLI])

* A relatively common disorder, condition, or mechanism

[a] [TLI] has been shown to increase mildly after food intake. The same change has not been documented for [PLI].

(2) Using decision thresholds based on receiver operating characteristic (ROC) analysis (not URLs), diagnostic specificity for severe pancreatitis is high (about 90–100 %), but diagnostic sensitivity for pancreatitis has been reported to be no higher than about 30–50 % (less than for serum lipase activity), and even lower for chronic pancreatitis, which may be associated with reduced acinar tissue, decreased [TLI], and EPI.[19]

(3) Dexamethasone (0.25 mg/kg per os daily) was associated with increased [TLI] by day 7 of treatment and was WRI again 7 d after withdrawal.[20] The mean increase was three times baseline mean, but only 2 of 12 dogs had [TLI] > the URL. The authors suggested that the [TLI] was increased because of direct effects of dexamethasone on the pancreas, but the cause is unknown.

c. Cats: A moderately to markedly increased serum [TLI] in a nonazotemic cat is supportive of pancreatitis, but cobalamin deficiency should also be considered, and pancreatitis may be present without an increased serum [TLI].

(1) In an experimental feline pancreatitis study, serum [TLI] increased the first day but was mostly WRI on day 2, while [PLI] remained increased until day 4 compared to sham controls (which also had increases for several days).[21]

(2) The diagnostic sensitivity of [TLI] is much better for moderate to severe pancreatitis than for mild pancreatitis.[22] In various studies, diagnostic specificities have varied from about 30 % to 80 % overall.[22–25]

(3) In a prospective study involving 28 cats with clinical signs compatible with pancreatitis, there were not significant differences in serum [TLI] among cats without pancreatic lesions (10), cats with pancreatitis and fibrosis (9), cats with pancreatic fibrosis but without active inflammation (4), and cats with acute necrotizing pancreatitis (5).[25] Using the correct URL for the assay (the URL was reported erroneously in the manuscript),[12] the [TLI] was increased in 50 % of cases with no pancreatic lesion and in only 40 % and 44 % of cases with acute necrotizing pancreatitis or fibrosis plus inflammation, respectively. Increasing the medical decision limit would have improved the diagnostic specificity while yielding a poor diagnostic sensitivity of 33 %. Despite efforts to sample lesions and evaluate multiple sites histologically, focal inflammatory or necrotic lesions might not have been sampled, or there might have been acinar cell injury without detectable lesions.

d. Horses that have strangulating intestinal obstruction and endotoxic shock can have increased plasma [trypsin] (as measured by an activity assay).[26] The trypsin may be released from pancreatic acinar cells because of poor perfusion of splanchnic tissues. Similarly, in a follow-up study involving ten horses, some with strangulating intestinal obstructions had increased plasma [TLI], but those with nonstrangulating intestinal obstructions did not.[27] It is not known whether the increased plasma [TLI] was due to increased release of pancreatic TLI molecules, whether more TLI molecules were bound to proteins (e.g., acute-phase proteins), or whether TLI molecules gained access to plasma via damaged intestines.

2. Stimulation by food intake or cholecystokinin and secretin administration causes mild or mild–moderate increases in [TLI].[28,29] Intravenously administered secretin and cholecystokinin caused a mean 3.4× increase in [TLI] in healthy dogs –

from 7.4–12.6 μg/L (mean of 10.3 μg/L) to 13.0–114.8 μg/L (mean of 35.0 μg/L) – but [TLI] remained < 2.5 μg/L in 6 dogs with EPI.[29]

B. Decreased renal clearance in disorders that cause decreased GFR
   1. Trypsinogen is cleared from plasma by kidneys. Disorders that cause a decreased GFR may cause an increased [TLI].[30,31]
   2. With decreased GFR from prerenal, renal, or postrenal disorders (see Chapter 8, Azotemia and Uremia, Section II.B), concurrent azotemia is expected.

C. Cobalamin deficiency in cats
   1. Although cats with EPI (and decreased [TLI]) often have hypocobalaminemia, cobalamin deficiency has been associated with increased [TLI] in some cats. Of 19 cats with severe cobalamin deficiencies associated with gastrointestinal disease, 15 had increased serum [TLI] at the time of diagnosis; 10 had a [TLI] > 100 μg/L (RI = 12–82 μg/L). The reasons for the increased [TLI] in these cats were not determined. Serum [TLI] decreased in 9 of the 19 cats when they were given parenteral cobalamin therapy.[32]
   2. Of nine cats with various types of inflammatory bowel disease, five had mild to moderate decreases in [cobalamin]. One of those five had a greatly increased [TLI] of undetermined cause.[33]

D. Although serum [TLI] was greater in dogs on a high-protein diet compared to dogs on a low-protein diet, the mean concentrations for all groups were within common canine RIs.[34]

IV. Decreased [TLI] (Table 15.3)

A. Decreased release from pancreatic acinar cells
   1. Chronic pancreatitis that leads to destruction of most acinar cells[5]
   2. Pancreatic acinar atrophy in dogs

B. In dogs with clinical signs of maldigestion (due to EPI) or malabsorption (due to small intestinal disease), the diagnostic sensitivity, specificity, and accuracy of a fasting serum [TLI] for EPI are very high (approaching 100 %).[35] However, the presence of active pancreatitis[15] or a disorder that is causing decreased GFR may increase [TLI] enough to mask a deficient state.

C. A decrease in serum [TLI] without clinical disease or signs of EPI may reflect subclinical disease. Of 20 German shepherds and rough-coated collies with decreased [TLI] but no clinical disease, seven developed clinical EPI within 6–46 mo.[36]

D. Cats with decreased serum [TLI] and clinical signs suggestive of EPI (e.g., weight loss, soft or voluminous stool, and polyphagia) have had additional diagnostic evaluations, responses to enzyme replacement therapy (often with cobalamin supplementation), or both that supported diagnoses of EPI.[6,37]

Table 15.3. Disorders and conditions that cause decreased [TLI] in dogs and cats

Decreased release of trypsinogen (or trypsin) from the pancreas
Chronic pancreatitis*
Pancreatic acinar atrophy*

* A relatively common disorder, condition, or mechanism

PANCREATIC LIPASE IMMUNOREACTIVITY (PLI) CONCENTRATION IN DOGS AND CATS

I. Physiologic processes (Figure 15.1)

II. Analytical concepts
  A. Terms and units
    1. *Pancreatic lipase immunoreactivity* (PLI) reflects the concentration – not activity – of pancreatic lipase protein ($M_r \cong 51{,}000$ in dogs and 52,000 in cats); the protein is detected by use of species-specific antibodies (hence immunoreactivity).
    2. Because immunologic PLI assays are species specific, the species name is commonly included to describe the assay and result: canine pancreatic lipase immunoreactivity (cPLI), canine pancreatic lipase (cPL) concentration, feline pancreatic lipase immunoreactivity (fPLI), or feline pancreatic lipase (fPL) concentration.
    3. Quantitative and semiquantitative results are reported in units of mass concentration: μg/L.
    4. With a qualitative point-of-care test, results are considered "normal" (WRI) if the sample reaction spot is lighter than the reference spot, or "abnormal" (increased [PLI]) if the intensity of the sample reaction spot is the same as or greater than that of the reference spot.[38] A company white paper adds that "abnormal" results are considered "elevated" if the sample reaction spot is the same color as the reference spot, or "consistent with pancreatitis" when the sample reaction spot is darker than the reference spot and therefore likely > 400 μg/L.[39] (Note: No result is inconsistent with pancreatitis; [PLI] may not be increased in pancreatitis.)
  B. Assays
    1. RIA and ELISA methods have been developed for canine and feline [PLI].[40] They are designed to measure the concentration of pancreatic lipase protein bound by species-specific polyclonal or monoclonal antibodies and are thus different from assays that measure lipase activity (units = U/L; see Chapter 12, Basic Principles in Clinical Enzymology, Section IV).
    2. Canine and feline assays were initially developed as RIAs[41,42] and subsequently as ELISAs, all using rabbit polyclonal antibodies raised against lipase purified from canine or feline pancreas.[40] The canine antibodies were shown to localize only to pancreatic acinar cells in immunohistochemical studies of canine tissues; reports of similar studies were not found for cats. Lack of transient increases in canine or feline serum [PLI] after heparin administration supports that the antibodies do not interact with lipoprotein lipase.[43]
    3. The initial ELISA was later modified to yield current canine and feline quantitative (ELISA)[44] and point-of-care screening tests that use mouse monoclonal antibodies developed against species-specific recombinant peptide antigens.[45]
      a. With a minority of samples, diagnostic classification regarding positivity differs among these assays;[46,47] the ELISA is considered to be the more reliable assay.
      b. The screening test was designed to yield a positive result when [PLI] was at the URL of the ELISA, which is lower than the decision limit for pancreatitis.[47] Follow-up with the ELISA can be used for quantitation and further assessment.
    4. Other point-of-care canine PLI immunoassays have since been developed, each with its own limitations.[48,49]

5. Analytical differences among the various historical and current PLI assays are reflected by substantial (as much as 50 %) proportional biases and the consequent use of substantially different assay-specific reference values and diagnostic decision thresholds.[44,50] These differences and varied criteria for diagnosing pancreatitis, often lacking the "gold standard" of pancreatic histology, complicate interpretation of published PLI data.

C. Sample: Serum is the preferred sample for blood testing; PLI has also been assessed in peritoneal fluid.[51]

D. Severe lipemia might cause false increases or decreases in [PLI] if not cleared prior to analysis, and lipemia promotes in vitro hemolysis, which may interfere with analysis of PLI or other tests for which the sample was submitted (e.g., [folate]). An interference study of a canine ELISA showed mild effects of lipid, hemoglobin, and bilirubin when added to samples with low- to mid-range [PLI]. However, some details of the study were not reported, and differences were of magnitudes unlikely to change classification of results.[44]

III. Increased [PLI] (Table 15.2)

A. Increased release from damaged pancreatic acinar cells

1. During experimental and spontaneous pancreatitis, lipase is released from damaged pancreatic acinar cells and enters the blood (probably via lymphatic vessels). Spontaneous pancreatitis is commonly idiopathic, but it may be associated with other disorders such as pancreatic neoplasms, which typically induce inflammation that promotes cell damage and lipase release.[52,53] With pancreatic acinar cell carcinomas, increased secretion of lipase by neoplastic acinar cells cannot be excluded as a contributor to hyperlipasemia.[54]
2. Dogs: Several studies have shown [PLI] to have greater diagnostic sensitivity and specificity for pancreatitis than do results of other blood tests.[40] However, with an appropriate decision limit, lipase activity assays using 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester have performed similarly.[55,56] The sensitivity of [PLI] for mild or chronic pancreatitis has been lower than that of certain enzymatic lipase[9] or α-amylase assays,[19] but PLI assays have the advantage of greater analytical specificity for pancreatic lipase and their results have greater diagnostic specificity for acute pancreatitis. In one study, the diagnostic specificity and sensitivity of increased [PLI] for histologically moderate or severe canine pancreatitis were > 90 %.[54]
3. Cats: [PLI] (using a decision threshold greater than the URL) is reported to have better diagnostic sensitivity for pancreatitis than does [TLI] in cats, with sensitivity being lower for mild acute or chronic pancreatitis than for pancreatitis with moderate to severe acute and chronic changes.[22] After experimental initiation of acute pancreatitis in cats, [PLI] increased more than [TLI] (50 × baseline versus 35 × baseline) and remained increased longer (10 d vs 3 d).[57] [PLI] has also increased in cats with pancreatic trauma caused by falls from buildings.[58,59]

B. Decreased renal clearance

1. The role of the kidneys in clearance of canine and feline pancreatic lipase is not clear. Renal disease or other causes of decreased GFR may have similar or different effects on [PLI] and serum lipase activity (see Chapter 12, Lipase, Section V.B).
2. In a study using an ELISA, [PLI] was mildly increased in 16 of 17 dogs with experimentally induced chronic renal failure, but not above the suggested diagnostic threshold for pancreatitis; the same dogs did not have increased serum lipase activity. One dog had extreme increases in [PLI] and serum lipase activity but was considered an outlier.[60] Because serum lipase activity increased with experimental renal failure in

previous canine studies but not in this study, it is not clear if the model could appropriately assess changes in [PLI] caused by renal failure.

3. In a small study of reversible gentamicin-induced acute renal failure in dogs, increased [PLI] (and lipase activity) accompanied azotemia in a small percentage of samples, but there was not a consistent or clear effect.[61]
4. Renal disease was the most common disorder associated with hyperlipasemia and increased [PLI] in critically ill dogs, but hyperlipasemia correlated poorly with [creatinine].[56] It is not clear if hyperlipasemia was caused by renal disease or concurrent pancreatic disease.
5. Cats with experimentally induced chronic renal failure sufficient to increase [TLI] did not have increases in [PLI].[31] However, PLI samples had been stored at –80 °C for nearly 9 yr, 6 yr longer than the TLI samples and longer than feline PLI stability has been assessed (44 mo), thus providing opportunity for acute or chronic causes of sample deterioration.

C. Increased [PLI], including values over the decision limit for pancreatitis, sometimes occurs in dogs and cats without clinically suspected pancreatitis. This may reflect subclinical pancreatitis, other undefined alterations in the production or clearance of pancreatic lipase, or inaccurate results. Such unexpected apparent increases were documented in untreated healthy dogs assessed 14 times over 3 mo for biological variation of [PLI]; approximately 12 % of the values were greater than the URL,[62] despite the fact that appropriate RIs should encompass about 95 % of analytical and biological variations, with just 2.5 % of values from healthy individuals being high.
   1. Dogs: Conditions associated with increased [PLI] in the absence of clearly documented pancreatitis include hyperadrenocorticism,[63] diabetes mellitus,[64] treatment with potassium bromide or phenobarbital,[65] heart failure from mitral valve disease,[66] mushroom toxicosis,[67] gastric dilation and volvulus,[68] and postprandial hypertriglyceridemia in overweight dogs.[69]
   2. Cats: Increased [PLI] has been reported in many cats with inflammatory bowel disease[70] or diabetes mellitus,[71] and in cats with cholangitis.[72]
D. Glucocorticoid administration: In some studies, serial testing of healthy or ill dogs receiving at least anti-inflammatory doses of glucocorticoids have shown associated increases in [PLI] without other supporting evidence of clinical pancreatitis.[73–75] Most increases have been mild, but some have been great enough to change interpretation and suggest pancreatitis. The cause is not clear.

IV. Decreased [PLI]: In a study using an ELISA, all 25 dogs with EPI (as defined by having a decreased [TLI]) had decreased [PLI].[76] However, current PLI assays are used to detect increases; decreases may not be measurable and are not recognizable because currently provided reference values include 0 or do not provide a lower reference limit. Instead, decreased functional pancreatic acinar mass associated with chronic pancreatitis or pancreatic acinar atrophy is routinely assessed by [TLI].

COBALAMIN (VITAMIN B_{12}) CONCENTRATION IN DOGS, CATS, AND CATTLE

I. Physiologic processes (Figure 15.2)[77–79]
   A. Cobalamin, a water-soluble vitamin produced by microorganisms and found in food products of animal origin, must be included in the diet of dogs and cats.

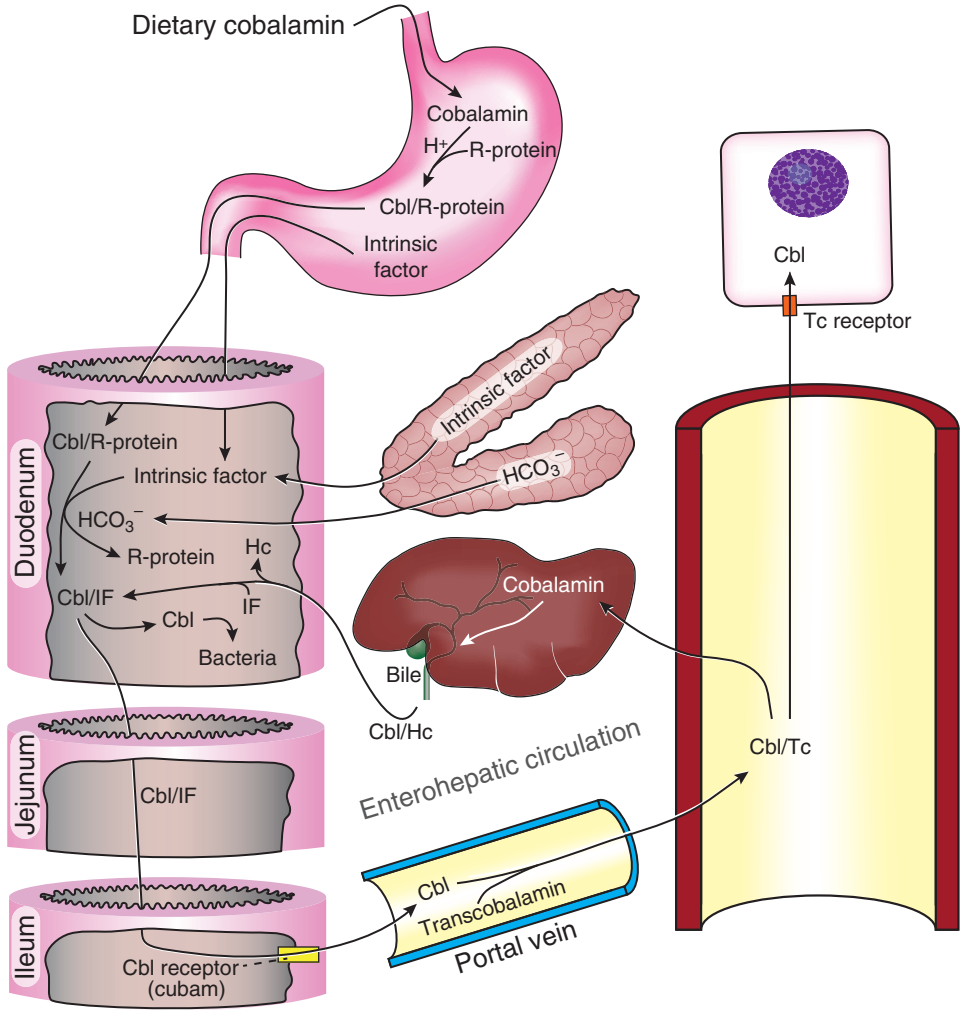


Figure 15.2. Physiologic processes that influence plasma or serum concentrations of cobalamin.

- Cobalamin enters the stomach via ingested foods and is released by pepsin digestion in the acidic environment. It binds to R-protein (R for rapid electrophoretic migration), which is a type of haptocorrin (also called cobalophilin) that is produced by salivary glands and the gastric mucosa.
- When this complex enters the duodenum, pancreatic proteases detach cobalamin, allowing it to bind to intrinsic factor secreted by pancreatic cells (dogs and cats) and the gastric mucosa (dogs). This transfer is promoted by an alkaline pH. Enteric bacteria use some of the cobalamin as it moves through the small intestine. When it reaches the ileum, the cobalamin/intrinsic factor complex binds to specific mucosal receptors, cubam receptors consisting of cubilin and amnionless, and enters enterocytes.
- When cobalamin enters the portal blood, it binds to transcobalamin (also called transcobalamin II) for transport in plasma as *holotranscobalamin*. In people, the majority of plasma cobalamin is instead bound to haptocorrin (has been called transcobalamin I and III), which is relatively unavailable to most cells but enters hepatocytes; it might be present to a lesser extent in dogs, but it has not been identified consistently in canine, feline, or ovine plasma.
- Plasma Cbl/Tc binds to specific transcobalamin receptors on cells throughout the body and enters by endocytosis to participate in cellular metabolic pathways. Cobalamin that enters hepatocytes may be stored, used for metabolic processes, or excreted in bile as a complex with hepatic haptocorrin.[80] When this biliary Cbl/Hc reaches the intestine, proteases detach cobalamin from Cbl/Hc complexes, and the cobalamin can be used by bacteria or bound to intrinsic factor and reabsorbed in the ileum, thus completing a cycle of enterohepatic circulation that conserves cobalamin. However, some cobalamin is also excreted in feces, and some plasma Cbl/Tc passes into the renal filtrate and is excreted in urine if it escapes megalin-mediated resorption in the proximal renal tubules (not shown).

Cbl, cobalamin; Hc, haptocorrin; IF, intrinsic factor; Tc, transcobalamin

B. Cobalamin is a required cofactor in the metabolic pathways involving folate.
C. Cobalamin is also a required cofactor for the conversion of methylmalonyl coenzyme A to succinyl coenzyme A. Without this conversion, plasma methylmalonate accumulates, methylmalonic acidemia and aciduria may occur, and a neurologic disease develops because of defective formation of neuronal lipids. Methylmalonate can be measured in serum or urine by gas chromatography-mass spectrometry, and such results may be useful for complete interpretation of serum [cobalamin] by providing an indication of the adequacy of intracellular cobalamin. Serum [cobalamin] does not necessarily reflect cellular cobalamin sufficiency, e.g., when receptor-mediated uptake into cells is impaired or when cell uptake is sufficient to maintain cell functions but not without lowering plasma [cobalamin]. Hypocobalaminemic dogs without increased [methylmalonate] may not benefit from cobalamin administration, whereas increased [methylmalonate] in normocobalaminemic or even hypercobalaminemic dogs may reflect impaired intracellular cobalamin processing and a functional cobalamin deficiency.[81,82]
D. In people, most of the cobalamin measured by routine assays is bound to haptocorrin, which does not carry cobalamin to most target cells, so [cobalamin] does not reflect the cobalamin that is available to the tissues.[77] In contrast, transcobalamin appears to be the main cobalamin transporter in domestic mammals, with plasma haptocorrin playing little or no role in health, so serum or plasma [cobalamin] may better reflect cobalamin availability in domestic mammals.[83,84]
E. Based on a small 10-mo-long study of approximately 2-mo-old rescued stray kittens lacking evidence of significant clinical disease, serum [cobalamin] might be low in kittens relative to adults.[85] In a longitudinal study of healthy colony cats on a standardized and appropriate diet, serum [cobalamin] decreased about 33 % from age 3–4 yr to age 7–9 yr.[86]

II. Analytical concepts
A. Terms and units
1. *Cobalamin*, a contraction of "cobalt + vitamin," refers to a group of compounds that have a corrin (porphyrin-like) ring and various side chains bound to cobalt. The different side chains yield hydroxocobalamin, cyanocobalamin, methylcobalamin, and deoxyadenosylcobalamin, the latter two playing significant physiologic roles. The term *vitamin B_{12}* is used to refer to all of these forms collectively, and it commonly refers specifically to cyanocobalamin, the principal form produced for use in food and nutritional supplements.
2. Unit conversion for cyanocobalamin: pg/mL × 0.7378 = pmol/L; ng/dL × 7.378 = pmol/L (SI unit)[87]
B. Assays
1. Competitive protein-binding assays have largely replaced bioassays, and chemiluminescent assays have largely replaced immunoradiometric assays. These newer assays are typically designed to measure [cyanocobalamin] after conversion of physiologic cobalamin to cyanocobalamin by the addition of potassium cyanide.
2. With competitive protein-binding assays, cobalamins are typically freed from their binding proteins by alkaline hydrolysis and the addition of molecules (e.g., cobinamide, dithiothreitol) that help dissociate the complex and also block rebinding. Insufficient dissociation falsely lowers the measured [cobalamin]. If there are antibodies in the sample that can bind to porcine intrinsic factor (e.g., in human pernicious anemia), they can lead to falsely increased cobalamin values if they are not completely denatured.

3. In common clinical assays, released sample cobalamin and immobilized or labeled assay cobalamin compete for porcine intrinsic factor; the more cobalamin there is in the sample, the less porcine intrinsic factor will bind to the assay cobalamin and be detected. If the cobalamin-binding reagent contains contaminant cobalamin-binding proteins, they can bind to inactive cobalamin metabolites and give falsely increased cobalamin values.[88]
4. Results are compared to those for calibration standards; the type of cobalamin molecule used as a standard can significantly affect the results.

C. Sample
1. Serum is recommended and typically used. Data were not found regarding use of anticoagulated samples in domestic mammals.
2. Samples are stable at 8 °C overnight and up to 8 wk at −20 °C. The [cobalamin] was stable in canine and feline samples stored at 6 °C for 5 d, whereas there was a mild but significant decrease in [cobalamin] when stored at room temperature while exposed to daylight (-6 % at 24 h and 15 % after 5 d).[89]
3. Excessive exposure of samples to light may falsely decrease [cobalamin], but routine (several hours) sample exposure to laboratory fluorescent lighting does not appear to cause a diagnostically significant change.
4. [Cobalamin] measured in fasting and postprandial (1, 2, 4, and 8 h) samples from a small group of clinically healthy dogs showed no association of [cobalamin] with the presence of precentrifugation lipemia (samples were not tested until lipemia was reduced by centrifugation).[14] There was a statistically significant decrease in median [cobalamin] at 4 h and 8 h after baseline, but there was no consistent pattern of change, and few changes were of a magnitude likely to affect interpretation of patient results.
5. If samples collected for [cobalamin] are also to be tested for [folate], [PLI], or [TLI], as is commonly the case, preanalytical factors affecting each test should be considered.

III. Hypercobalaminemia

A. Hypercobalaminemia is uncommon.[90] However, hypercobalaminemia may reflect significant disease, and disorders or conditions that could increase [cobalamin] should be considered when interpreting concentrations that are decreased or WRI (Table 15.4).

B. Disorders or conditions that cause hypercobalaminemia
1. Cobalamin supplementation: parenteral or oral (in contrast, cobalt chloride, which might also be given to enhance performance in horses, does not cause hypercobalaminemia)[91]
2. Hepatic disorders: Because hepatocytes are a storage site for cobalamin, hepatocellular damage or necrosis could increase serum [cobalamin]. The magnitude or duration of the increase is not known. In addition, decreased functional hepatic mass or

Table 15.4. Disorders and conditions that cause hypercobalaminemia

Cobalamin supplementation (oral or parenteral)
Hepatocellular damage (Table 12.4)
Decreased hepatocyte uptake of cobalamin from blood
Hepatic dysfunction (decreased functional hepatic mass)
Portosystemic shunts
Neoplasms that increase synthesis of plasma cobalamin carrier proteins (potentially)

expression of cobalamin membrane receptors could decrease uptake of plasma cobalamin bound to either haptocorrin or transcobalamin, thus promoting increased serum [cobalamin].

3. Portosystemic shunts: Hypercobalaminemia was reported in 9 of 10 dogs with extrahepatic portosystemic shunts.[92] This might be caused by decreased hepatic uptake of cobalamin. Two of the four dogs with increased serum [methylmalonic acid] at the time of diagnosis were hypercobalaminemic, suggesting a functional cobalamin deficiency. It is not clear why hypercobalaminemia persisted at least 3 mo in some dogs; details of cobalamin testing and quality of RIs were not reported.
4. Neoplasia: Hypercobalaminemia in people has been attributed to certain hematologic (e.g., chronic myeloproliferative) and solid tissue neoplasms,[93] the former related mostly to increased production of haptocorrin by mature and precursor myeloid cells, and the latter related mostly to increased production of transcobalamin, either by neoplastic cells or other cells induced by the neoplasm.[93] Evidence for hypercobalaminemia caused by neoplasms in domestic mammals is currently minimal.[90,94]

IV. Hypocobalaminemia (Table 15.5)
   A. Serum [cobalamin] will not be decreased until the body reserve is depleted. Depletion typically occurs because of decreased ileal absorption of cobalamin, but the primary defect may be preabsorptive (i.e., before cobalamin absorption by the ileum).
   B. Disorders or conditions that reduce cobalamin absorption in the intestine
      1. Cobalt deficiency: Dietary cobalt deficiency in cattle can cause hypocobalaminemia; cobalt is needed for synthesis of cobalamin by ruminal bacteria.[95] The cobalamin deficiency leads to an increased plasma [homocysteine] because methylcobalamin is needed for the methylation of homocysteine to form methionine (Figure 15.3).
      2. Preabsorptive defect[79]

Table 15.5. Disorders and conditions that cause hypocobalaminemia

Cobalt deficiency in cattle
Preabsorptive defect in dogs and cats
EPI: pancreatic atrophy, chronic pancreatitis
Intestinal dysbiosis: EPI, impaired gastric acid secretion, enteric disorders (see the text)
Defective absorption of cobalamin in ileum of dogs and cats
Ileal disease:* inflammation, ileal resection, villous atrophy (viral, hypersensitivity, cytotoxic drugs)
Congenital receptor deficiency (Australian shepherds, giant schnauzers, border collies, beagles, komondor, and a beagle-mix)
Severe cobalamin deficiency in a cat (probable congenital malabsorption defect)
Unknown or multifactorial mechanisms
Pregnancy, especially late term (dogs)
Canine parvoviral enteritis
Feline hyperthyroidism

* A relatively common disorder, condition, or mechanism
Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

a. EPI[96,97]
   (1) Decreased secretion of pancreatic proteases and HCO_3^--rich fluid into the duodenum impairs cobalamin release from haptocorrin and cobalamin binding to intrinsic factor.
   (2) Decreased pancreatic intrinsic factor (especially in cats because they apparently lack gastric intrinsic factor) yields fewer cobalamin/intrinsic factor complexes for absorption.
   (3) [TLI] is commonly assessed with cobalamin; concurrence of a decreased [TLI] and hypocobalaminemia supports EPI.

b. *Small intestinal dysbiosis*, a change in the types or numbers of intestinal microorganisms, can lower serum [cobalamin] by increasing the amount of cobalamin bound to and perhaps utilized by intestinal bacteria, thereby decreasing the amount of cobalamin that is free for absorption. Hyperfolatemia concurrent with hypocobalaminemia is supportive of intestinal dysbiosis, so [folate] is typically assessed concurrently with [cobalamin]. Some healthy dogs developed mild to moderate hypocobalaminemia for at least 2 wk after being administered a commercial probiotic containing *Enterococcus faecium* SF68; folate changes were equivocal.[98]

3. Defective absorption of cobalamin in the ileum of dogs and cats
   a. Disorders and conditions that impair ileal mucosal function: inflammation (inflammatory bowel disease), villous atrophy, treatment with cytotoxic drugs, intestinal lymphoma, and ileal resection
   b. Congenital selective intestinal cobalamin malabsorption occurs in several breeds of dogs because of mutations in the genes for cubam, the ileal receptor for cobalamin-intrinsic factor complexes. Mutations have been identified in both cubam subunit genes: amnionless (Australian shepherds, giant schnauzers) and cubilin (border collies, beagles, beagle mix, and komondor).[99–105] A similar disorder appears to occur in Chinese shar peis, although a specific mutation has not been reported.[106,107] Affected dogs are developmentally delayed and have methylmalonic acidemia/aciduria, and dyshematopoiesis in addition to hypocobalaminemia.
   c. Severe cobalamin deficiency in a cat with methylmalonic acidemia:[108] The cause of the cobalamin deficiency was not established, but clinical evidence supported a congenital defect in cobalamin absorption.
4. Unknown or multifactorial mechanisms
   a. Pregnant bitches may develop hypocobalaminemia, particularly in late pregnancy and with larger litter sizes and greater parity, but any impact on health has not been established.[109]
   b. Canine parvoviral enteritis: Hypocobalaminemia was present in nearly 60 % of dogs with parvovirus enteritis, but [methylmalonate] was not assessed for evidence of cellular deficiency.[110] Potential contributors include decreased intestinal absorption, exudation of cobalamin/transcobalamin, and increased utilization of cobalamin by proliferating hematopoietic and intestinal epithelial cells. Expression of transcobalamin receptors increases on proliferating cells, and transcobalamin has a plasma half-life of only 60–90 min in people.[111]
   c. Feline hyperthyroidism: Hypocobalaminemia in hyperthyroid cats may resolve with return to euthyroidism, or it may reflect a separate concurrent disorder.[112] In the few hypocobalaminemic hyperthyroid cats that were tested, serum [methylmalonic acid] was not increased, so there was no evidence of a cobalamin deficiency at the cellular level.

FOLATE CONCENTRATION IN DOGS AND CATS

I. Physiologic processes (Figure 15.3): Folates are important in single-carbon (methyl or methylene) transfers for the synthesis of DNA nucleotides and for the formation of certain amino acids. Cobalamin and folate metabolism are linked in a reaction in which a methyl group is transferred from 5-methyltetrahydrofolate to cobalamin. In the absence of cobalamin, the methyl group is trapped in 5-methyltetrahydrofolate, and the result is a functional folate deficiency. Because 5-methyltetrahydrofolate contributes to the total measured [folate], serum [folate] may not be decreased even though there is a functional folate deficiency (i.e., the manifestations of a folate deficiency are present).

II. Analytical concepts
   A. Terms and units
      1. As generally used, *folate* refers to a group of molecules (vitamin B_9) with a folic acid core and various numbers of substitution groups (e.g., methyl, formyl, glutamyl, and pteridine). Specifically, *folate* (pteroylglutamate) is the anionic form of folic acid (pteroylglutamic acid), which is principally a synthetic molecule that is not present in significant amounts in natural foods; it must be enzymatically converted to tetrahydrofolate to be functional.
      2. Plasma contains only folate monoglutamates, mostly 5-methyltetrahydrofolate, whereas long-chain polyglutamates predominate in RBCs.
      3. Unit conversion for 5-methyltetrahydrofolate: ng/mL × 2.177 = nmol/L; µg/dL × 21.77 = nmol/L (SI unit);[87] a conversion factor of 2.266 would replace 2.177 for folic acid, and a factor of 2.245 would be used for tetrahydrofolate conversion
   B. Assays
      1. Competitive-binding assays (sometimes combined with cobalamin assay) are most common and assess folate concentration by the degree to which binding of a labeled folate standard is inhibited by folate in a test sample. The competitive-binding agent in folate assays is β-lactoglobulin (milk folate binder), which binds principally with 5-methyltetrahydrofolate.[113] Affinity for β-lactoglobulin varies among folate standards and folate molecules. Assays may include steps for denaturation of endogenous binding proteins (by boiling or high pH) to release folate, or, for RBC assays, conversion of polyglutamate forms to the monoglutamate derivative.
      2. Bioassays[114] have been replaced mostly by chemiluminescence immunoassays using magnetic beads coated with antibodies that capture competing folate–protein complexes (patient vs labeled standard), yielding signals that are inversely proportional to sample [folate]. Additionally, mass spectrometry can be used to measure individual folate forms.
   C. Sample
      1. Serum is the preferred sample, though RBC [folate] can be measured to assess long-term folate status. Because RBCs have high [folate], even mild hemolysis may yield falsely increased values.[14] Because lipemia promotes hemolysis in vitro, lipemia is best avoided; to this end, samples are ideally collected after at least an 8-h fast. Also, small increases in [folate] may occur postprandially.[14] EDTA-plasma may be used in some assays.
      2. Serum [folate] is stable at 4 °C for 1–3 d and at −20 °C for at least 4 wk; it is relatively unstable at room temperature.
      3. Folate is degraded by ultraviolet light, so avoiding excessive exposure of samples to direct light is recommended, but routine sample exposure to indoor lighting did not cause diagnostically significant decreases in [folate] with human samples.[115]

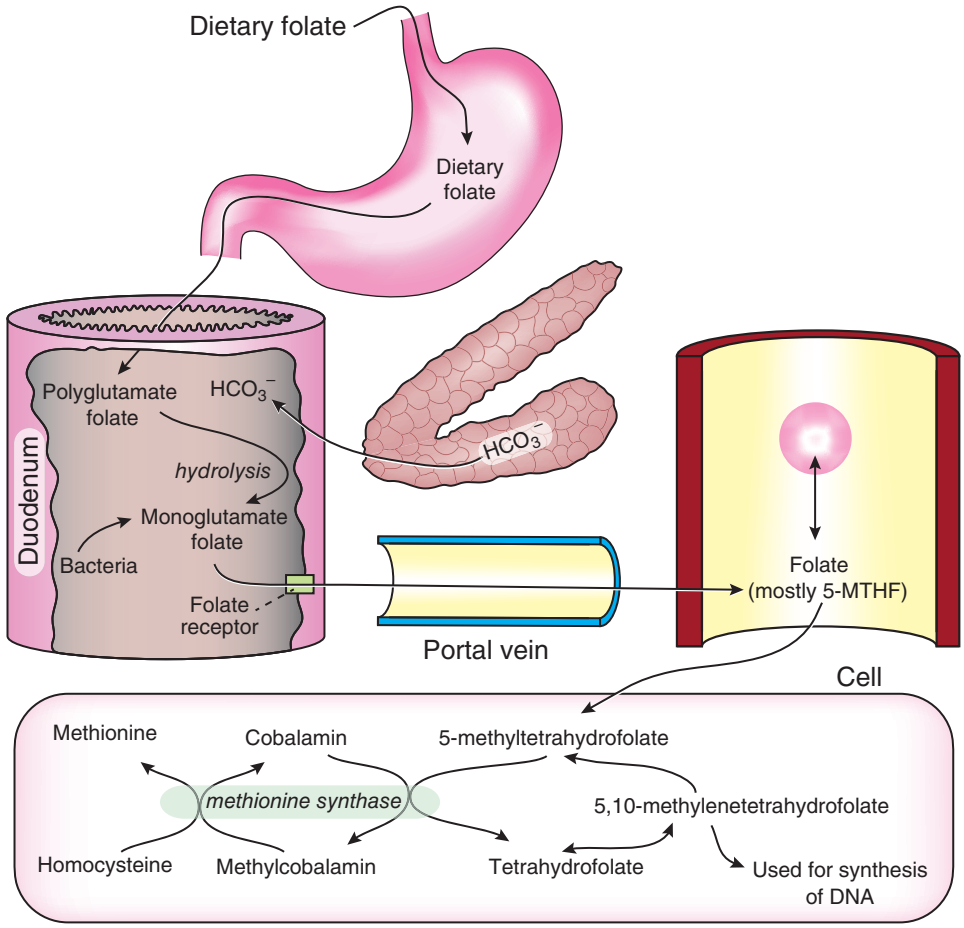


Figure 15.3. Physiologic processes that influence plasma or serum concentrations of folate and the cellular relationship of cobalamin and folate.

- Folate (from the Latin *folium* meaning "leaf") is a water-soluble vitamin present in food (e.g., green leafy plants) in a polyglutamate form. After digestion releases it from food, polyglutamate folate is hydrolyzed to monoglutamate folate at the brush border of the proximal small intestine by gamma-glutamyl hydrolase (EC 3.4.19.9, also known as folate conjugase). After receptor binding and cellular uptake by folate transporters, it is converted to the 5-methyltetrahydrofolate form, which enters the blood and is transported to tissues for biochemical reactions. Enteric bacteria can also produce folate.
- Cobalamin is a required cofactor for methionine synthase, which catalyzes the conversion of 5-methyltetrahydrofolate (the primary molecule in plasma) to tetrahydrofolate, which is then available for DNA synthesis in cells. If there is a cobalamin deficiency, the methyl group of 5-methyltetrahydrofolate is not transferred to cobalamin (methyl trapping), a cellular deficiency in tetrahydrofolate occurs, and DNA synthesis is impaired.

5-MTHF, 5-methyltetrahydrofolate

Table 15.6. Disorders and conditions that cause hyperfolatemia
Increased absorption of folate in small intestine
Intestinal dysbiosis: EPI, impaired gastric acid secretion, enteric disorders (see the text)
Low intestinal pH: EPI, excessive gastric acid production
High dietary intake
Parenteral supplementation
Cobalamin deficiency in cats (folate trapping)

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Hemolysis may give falsely increased values.

III. Hyperfolatemia (Table 15.6)
   A. Increased absorption of folate in the small intestine
      1. Small intestinal dysbiosis: Excessive folate produced by enteric bacteria is absorbed in the small intestine. The altered intestinal microbiota could be secondary to EPI or caused by impaired gastric acid secretion, decreased intestinal peristalsis, a defective mucus barrier, or decreased production of immunoglobulin by intestinal lymphoid cells.
      2. Low intestinal pH: Maximal folate absorption occurs in an acidic environment. Decreased intestinal pH (increased absorption) may occur with EPI (impaired HCO_3^- secretion) or excessive gastric acid production.
      3. High dietary intake: Folate absorption is mediated by intestinal folate receptors and transporters that can increase absorption with high intestinal concentrations of folate or supplemental folic acid.
   B. Parenteral supplementation: The magnitude and duration of hyperfolatemia would depend on dosage. A complete history should indicate whether this process is a potential cause of the abnormality.
   C. Cobalamin deficiency in cats:[32] 5 of 19 cats with severe cobalamin deficiencies had hyperfolatemia. When given parenteral cobalamin therapy, the [folate] decreased significantly in the 19 cats. Cobalamin deficiency may have decreased methionine synthase activity and therefore the formation and utilization of tetrahydrofolate via methyl trapping (see Figure 15.3), thus causing accumulation of the nonfunctional 5-methyltetrahydrofolate form of folate in plasma.
   D. In vitro hemolysis can cause falsely increased serum [folate] because RBCs contain folate.

IV. Hypofolatemia (Table 15.7)
   A. Decreased absorption of folate in the small intestine
      1. Diseases of the proximal small intestinal mucosa can cause impaired absorption of folate.

Table 15.7. Disorders and conditions that cause hypofolatemia
Decreased absorption of folate in small intestine
Small intestinal mucosal disease (proximal or diffuse)*
Dietary deficiency (rare)

* A relatively common disorder, condition, or mechanism

2. Dietary deficiency: Commercial diets should have more than minimum folate requirements, and thus folate-deficient diets are uncommon. Feeding a folate-deficient all-cheese diet to dogs caused hypofolatemia without apparent clinical consequences,[116] but two cats on a folate-deficient vegan diet were hypofolatemic and had macrocytic anemias that reportedly resolved after folic acid supplementation for 30 d.[117]

B. Other considerations
1. Prolonged phenytoin (Dilantin) treatment is known to lower serum [folate] in people. [Folate] was not significantly different in phenytoin-treated and nontreated dogs that were fed a folate-deficient diet.[116] The effect of phenytoin on [folate] is not known for dogs fed a folate-adequate diet.
2. Sulfasalazine also is reported to decrease folate absorption and lower [folate] in people, but similar findings have not been reported for dogs or cats.[118]
3. In theory, sterilization of the intestine with antibiotics may lower serum [folate] because a major source of folate (intestinal flora) would be removed. Also, folate is involved in DNA synthesis, so extensive neoplastic cell growth could potentially deplete folate levels. Hypofolatemia was more common in dogs with gastric carcinoma (8 of 15) than in dogs with chronic gastritis (4 of 28) or healthy dogs (1 of 7), but the cause of this difference was not determined.[119]

INTERPRETATION OF CONCURRENT TLI, PLI, COBALAMIN, AND FOLATE DATA

When cobalamin and folate are assessed because of suspected intestinal disease, TLI is typically assessed concurrently so that primary pancreatic disease and primary intestinal disease can be differentiated. PLI is often assessed concurrently with folate and cobalamin because acute and chronic pancreatitis and intestinal malabsorptive disorders may have similar clinical signs (vomiting, diarrhea, abdominal pain) and can occur together. Common panel patterns are provided in Table 15.8.

Table 15.8. Expected cobalamin, folate, TLI, and PLI findings with selected intestinal and pancreatic disorders in dogs and cats

Disorder	[Cobalamin]	[Folate]	[TLI]	[PLI]
Malabsorption				
Proximal small intestine	WRI	↓	WRI	WRI–↑[a]
Distal (ileal) small intestine	↓	WRI	WRI	WRI–↑[a]
Diffuse small intestine	↓	↓	WRI	WRI–↑[a]
Intestinal dysbiosis	↓	↑	WRI	WRI
EPI	↓–WRI	↑–WRI	↓	WRI[b]
Acute pancreatitis	WRI	WRI	↑[c]	↑
Pancreatitis[d] + dysbiosis	↓–WRI	↑	↑[c]	↑

[a] [PLI] may be increased with intestinal disease when there is concurrent pancreatic inflammation.
[b] Although [PLI] may decrease in EPI, decreases cannot be detected by current assays.
[c] [TLI] stays increased for a shorter time than [PLI], so [TLI] may be WRI when [PLI] is increased.
[d] Dysbiosis is more commonly associated with chronic pancreatitis, but clinical findings may support acute pancreatitis.

α_1-PROTEINASE INHIBITOR (α_1-PI) CONCENTRATION IN DOGS AND CATS

I. Physiologic processes
   A. *α_1-PI*, also called α_1-protease inhibitor, α_1-antiproteinase, α_1-antitrypsin, or SERPINA1, is an α_1-globulin (protein) that inhibits the activity of serine proteases (proteinases) including trypsin, chymotrypsin, and neutrophil elastase. It is distinct from the related serine proteinase inhibitor α_1-antichymotrypsin (SERPINA3).
   B. Although characterization has been incomplete, proteins isolated from several species have been considered α_1-PI based on antitrypsin or anti-elastase activities, amino acid sequences, and molecular mass (M_r = 54,500–59,000 for dogs, cats, and horses; M_r = 52,000 for human α_1-PI).[120,121]
   C. Because α_1-PI has nearly the same molecular mass as albumin, it may mirror albumin leakage into the intestine. In health, α_1-PI is present in plasma, interstitial fluid, and lymph but not in the intestinal lumen.[122]
   D. α_1-PI can persist and be measured immunologically in intestinal contents because it resists degradation by inhibiting the proteinases that could destroy it.
   E. The major site of α_1-PI synthesis is hepatocytes, with contributions from other cells, including certain enterocytes, leukocytes, and pneumocytes.

II. Analytical concepts
   A. α_1-PI can be measured in serum, plasma, or feces as an indicator of intestinal protein loss. Serum, as opposed to plasma, is typically used in veterinary medicine. A protein-losing enteropathy is expected to promote increased fecal [α_1-PI] and decreased serum [α_1-PI].
   B. Assays for fecal and serum α_1-PI are immunoassays that use species-specific antibodies to recognize α_1-PI protein. The result may therefore be referred to in species-specific notation as, for example, cα_1-PI for canine α_1-PI.
   C. Fecal α_1-PI
      1. Unit: µg/g of feces
      2. Dogs: The original canine fecal α_1-PI assay, an ELISA,[123] was replaced by a RIA that uses rabbit polyclonal antibodies raised against α_1-PI purified from canine serum.[124] Differences in the assays must be considered when interpreting published data.
      3. Cats: An ELISA for feline fecal α_1-PI that uses rabbit polyclonal antibodies raised against α_1-PI purified from feline serum has been developed.[125]
      4. Because of sample-to-sample variations in fecal [α_1-PI], three separate voided fecal samples are collected. Collection of feces by digital or mechanical removal of feces may introduce blood into the sample, which can result in falsely increased [α_1-PI].[126]
      5. After collection, the fecal samples should be immediately frozen and shipped with dry ice to the reference laboratory. When kept at 37 °C for 72 h, the [α_1-PI] decreased by about 30 %; when kept at 4 °C for 72 h, the concentration decreased by about 5 %.[123]
      6. The potential contribution of enterocytes or intestinal inflammatory cells to fecal [α_1-PI] is unknown.
   D. Serum α_1-PI
      1. Unit: mg/L; 1 mg/dL = approximately 0.18 µmol/L (the conversion factor varies slightly with differences in M_r among species)
      2. RIAs to measure serum [α_1-PI] with rabbit polyclonal antibodies have been developed for use in dogs and cats,[121,125, 127] but they have not yet been commercially available.

3. A mean decrease in [α_1-PI] of 11 % was reported for serum from healthy dogs when the serum was stored for 1 yr at –20 °C.[127]

E. If protein-losing enteropathy leads to decreased serum [α_1-PI], fecal [α_1-PI] may not increase as much as expected simply because of less α_1-PI in plasma to leak into the intestine. Therefore, for some conditions, the ratio of serum [α_1-PI] to fecal [α_1-PI] may yield better diagnostic value than either test alone.[128]

III. Increased fecal [α_1-PI] (Table 15.9)

A. Increased plasma protein loss into intestines

1. Protein-losing enteropathies caused by extensive intestinal disease such as intestinal lymphangiectasia, idiopathic inflammatory bowel disease (including lymphocytic, plasmacytic, or eosinophilic disorders), intestinal lymphoma, intestinal carcinoma, histoplasmosis, intestinal parasitism, and parvoviral enteritis[122,129, 130]
2. Blood loss into the alimentary tract: Blood loss may be associated with protein-losing enteropathies (e.g., ulceration or carcinoma), but it may also be due to hemorrhage from nonenteric disease (i.e., thrombocytopenia, coagulopathy, or gastric ulceration). In a study involving seven dogs that were being treated with the nonsteroidal anti-inflammatory agents meloxicam or carprofen, fecal [α_1-PI] did not increase.[131]
3. Contamination of feces with blood or plasma during collection of the fecal sample may cause or contribute to increased [α_1-PI].
4. Young dogs, especially those less than 6 mo of age, had greater median and maximum [α_1-PI] than dogs > 1 yr of age, though subclinical disease may have contributed to the reported differences.[124]

B. Increased fecal [α_1-PI] may be more sensitive to at least some early protein-losing enteropathies than are serum albumin and total protein concentrations.[132]

IV. Decreased serum [α_1-PI]

A. Protein-losing enteropathy: Preliminary assessments of serum [α_1-PI] suggest it may have diagnostic utility. Serum [α_1-PI] was decreased in 9 of 18 hypoproteinemic dogs with protein-losing enteropathy but in only 1 of 18 normoproteinemic dogs with chronic food-responsive diarrhea.[133]

B. Hypocobalaminemic Yorkshire terriers: Serum [α_1-PI] was also decreased in 25 % of 52 cobalamin-deficient Yorkshire terriers, a breed with a propensity for protein-losing enteropathy, while being WRI for all 60 normocobalaminemic Yorkshire terriers.

Table 15.9. Disorders and conditions that cause increased fecal [α_1-PI]

Increased plasma protein loss into intestines
Protein-losing enteropathies:* infectious or noninfectious inflammatory bowel disease, idiopathic or secondary intestinal lymphangiectasia, intestinal neoplasia, intestinal parasitism
Blood loss into the alimentary tract
Contamination of feces with blood
Young dogs, especially < 6 mo (when using adult reference values)

* A relatively common disorder, condition, or mechanism

Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

Hypoalbuminemia was associated with cobalamin deficiency in these dogs, supporting protein-losing enteropathy, but the retrospective study design did not provide definitive diagnoses or exclude possible effects of potentially concurrent renal (increased loss with proteinuria) or hepatic (decreased production with hepatic dysfunction) disease on serum [α1-PI].[134]

C. Proteinuria: Serum [α_1-PI] may decrease in some dogs with proteinuric renal disease.[135] However, evaluated samples were stored at –20 °C or –80 °C for a median of 2.2 yr (7.25 yr maximum) with no similarly stored samples from healthy dogs to control for storage conditions. Also, it was not clear why the RIs differed substantially from those used for a different study[136] that cited use of the same assay.[127]

D. Hepatic dysfunction: Although hepatic failure may cause decreased serum [α_1-PI] because of decreased synthesis,[135] this has not been clearly shown.

E. Severe inflammatory response syndrome (SIRS) or sepsis: At admission, approximately half of 19 dogs with noninfectious SIRS or sepsis had decreased serum [α_1-PI] when compared to a concurrently established new RI for the assay, and [α_1-PI] was inversely correlated with plasma [IL-6], suggesting the possibility of α_1-PI being a negative acute phase protein in dogs.[136] However, in a different study that made use of the same assay with a different RI, decreased serum [α_1-PI] was not apparent in dogs with inflammatory disease (pancreatitis, chronic hepatitis).[135]

F. Decreases in serum [α_1-PI] may be masked by concurrent corticosteroid administration, as serum [α_1-PI] was substantially increased in healthy beagles given oral hydrocortisone (8.5 mg/kg every 12 h). The increases, apparent by day 5 and progressive over approximately 2 mo of treatment, may relate to increased α_1-PI synthesis.[128]

D-XYLOSE ABSORPTION TEST IN DOGS, CATS, AND HORSES

I. Physiologic processes

A. *D-Xylose* is a simple pentose that is not commonly ingested, and very little is present in blood or liver. If ingested, it is passively absorbed in the duodenum and proximal jejunum, from which it enters portal blood. The degree to which it is taken up by hepatocytes and other cells in domestic species is not clear, but much of it passes through the glomerular filtration barrier and is excreted in urine.

B. In people, D-xylose enters the glucuronic acid–xylulose pathway in hepatocytes, so impaired hepatocyte function could alter D-xylose curves. It has usually been assumed that very little of the absorbed D-xylose is metabolized by hepatocytes or other cells of domestic mammals, but this assumption may be invalid given that only about half of the D-xylose administered to dogs was found in their urine.[137]

II. Analytical concepts

A. Unit conversion: mg/dL × 0.06661 = mmol/L (SI unit)[87]

B. Assays

1. Multiple D-xylose assays are available, but clinical laboratories do not commonly offer them.

2. A colorimetric assay using phyloroglucinol has been used for equine and feline sera.[138] Other assays involve deproteination of whole blood and analysis by gas chromatography-mass

spectrometry or enzymatic methods.[139,140] An o-toluidine assay can measure [D-xylose] based on differences in absorbances of the reaction products produced by hexoses (e.g., glucose) and pentoses (e.g., D-xylose).[141]

3. The capability of the intestine to absorb sugars other than just D-xylose (e.g., methylglucose, rhamnose, and lactulose) can be determined by quantitation using high-performance liquid chromatography or gas chromatography-mass spectroscopy.[140,142,143]

C. Sample
1. The preferred sample varies with the assay system. Serum is generally preferred. Urine and plasma have also been used.
2. Information on D-xylose stability was not found.

III. D-Xylose absorption tests
A. Basics of procedure
1. The *D-xylose absorption test* typically consists of administering D-xylose orally, collecting multiple timed blood samples, and graphing the [D-xylose] over time to generate a D-xylose curve that reflects intestinal ability to absorb D-xylose. Use of a single, timed post-D-xylose sample has also been evaluated in dogs.[140] The peak blood [D-xylose] and area under the [D-xylose] curve generally reflect the amount of D-xylose absorbed by the intestine.
2. Because much D-xylose is cleared from plasma by passing through the glomerular filtration barrier, the amount of D-xylose in urine also reflects the amount of D-xylose absorbed in the intestine. However, urine [D-xylose] is also affected by renal conservation of water.
3. Decreased GFR can affect absorption data by increasing serum or plasma [D-xylose] and decreasing renal clearance of D-xylose.

B. In dogs and cats
1. D-Xylose absorption tests have been used to evaluate small intestinal function.[144–146] In some animals with small intestinal disease, D-xylose was poorly absorbed, and thus the absorption curves were flat.
2. In most current textbooks, small animal internists no longer recommend D-xylose absorption tests, perhaps because of the limited availability of D-xylose assays or because other methods of characterizing intestinal disease suffice or are preferred.

C. In horses
1. D-Xylose absorption tests have been used to evaluate the absorptive function of the small intestine.[147–149]
2. Xylazine sedation administered minutes before D-xylose administration had little or no effect on D-xylose absorption in healthy horses.[150]
3. In addition to small intestinal mucosal disease, decreased feed intake and high-energy feed[147] have been shown to decrease intestinal absorption of D-xylose and depress the absorption curve. Maximal plasma [D-xylose] was lower and later in healthy mares after 72 and 96 h of feed deprivation.[151]
4. Compared to adult horses, D-xylose absorption is increased in young foals, becoming similar to adult D-xylose absorption at about 3 mo of age.[152]

IV. Disorders and conditions that alter xylose absorption curves (Table 15.10)

Table 15.10. Disorders and conditions that alter serum or plasma D-xylose absorption curves
Decreased area under the curve (flat absorption curve) = reduced intestinal D-xylose absorption
Small intestinal mucosal disease*
Intestinal overgrowth by D-xylose-metabolizing microorganisms
Incomplete delivery of D-xylose to intestine: vomiting, delayed gastric emptying
Rapid transit time: diarrhea
Diet (horses): high-energy concentrate, food deprivation
Increased area under the curve = increased[a] or persistent [D-xylose]
Decreased D-xylose excretion from decreased GFR
Possible: impaired hepatic function (less metabolism of D-xylose)

* A relatively common disorder, condition, or mechanism

[a] D-Xylose absorption is increased in young foals, becoming similar to adult D-xylose absorption at about 3 mo of age.

GLUCOSE ABSORPTION TEST IN HORSES

I. Physiologic processes and introductory concepts

A. The glucose absorption test is used primarily in horses that are presented because of chronic weight loss. The horses may be passing malformed feces (e.g., cow-pie). If the mucosal disease is limited to the small intestine, feces may have normal consistency.

B. Glucose is a simple hexose that is absorbed in the duodenum and proximal jejunum, from which it enters portal blood. A large portion of the absorbed glucose is removed from the portal blood by hepatocytes, and the remainder enters the systemic blood. From there, glucose may enter metabolic pathways of cells (e.g., glycolysis or glycogen synthesis) or be excreted by kidneys if the renal resorptive maximum for glucose is exceeded.

C. In horses, microbes of the large intestine digest carbohydrates and produce volatile fatty acids that are absorbed by the mucosa. Therefore, disorders of the large intestine in horses can cause clinical states in which ingested carbohydrates are malassimilated.

D. Whereas the results of the D-xylose absorption test depend primarily on the intestinal absorption of D-xylose, the results of the glucose absorption test depend on intestinal absorption and glucose utilization by hepatocytes and other tissues (primarily muscle and adipose tissue). However, there are three major advantages of the glucose absorption test compared to the xylose absorption test: (i) glucose is more readily available, (ii) glucose is less expensive, and (iii) assays are more readily available for measuring serum or plasma [glucose].

E. The glucose absorption test can be used to assess the intestinal absorption of monosaccharides in simple-stomach mammals but cannot be used to assess intestinal absorption in ruminants. It is rarely used in the evaluation of dogs and cats, probably for two reasons: (i) other diagnostic methods are available (e.g., those using folate and cobalamin), and (ii) disorders that cause glucose intolerance are more common. When used to assess carbohydrate tolerance, the procedure is called the *oral glucose tolerance test*.

II. Analytical concepts (see Chapter 14, Glucose Concentration in Serum, Plasma, or Whole Blood, Section II)

III. Oral glucose absorption test

A. Basics of procedure: Glucose is administered via a stomach tube, and then multiple blood samples are collected to assess the intestinal ability to absorb it. Assuming normal glucose

Table 15.11. Disorders and conditions yielding oral glucose absorption curves that fail to meet criteria of adequate glucose absorption in horses

Malabsorption due to small intestinal mucosal disease:* inflammation, neoplasia, villous atrophy
Altered glucose delivery to intestine (delayed gastric emptying, prolonged intestinal transit time)
Rapid transit time associated with diarrhea
Increased use of glucose by hepatocytes or other tissues

* A relatively common disorder, condition, or mechanism

Note: States of insulin resistance (equine metabolic syndrome, diabetes mellitus) may decrease cellular glucose uptake and therefore alter intestinal glucose absorption results.

metabolism, the peak blood [glucose] should reflect the amount of glucose absorbed by the intestine.

B. Procedure[153]
   1. Feed is withheld overnight.
   2. A blood sample is collected for a baseline (or 0-time) [glucose].
   3. Glucose (1 g/kg body weight as a 20 % solution, weight/volume) is administered via stomach tube.
   4. Blood samples are collected at 30, 60, 90, 120, and 180 min after glucose is administered.
   5. Blood samples are processed appropriately, and glucose concentrations are measured in the serum or plasma samples.

C. Expected values in healthy horses
   1. Two criteria have been used as evidence of adequate intestinal absorption of glucose: [glucose] at 120 min is more than either 85 %[154] or 100 %[155] above the baseline [glucose].
   2. The guidelines are based on data from 11 healthy horses. The peak [glucose] at 120 min was approximately 2 × baseline, i.e., 178 ± 29 mg/dL at 120 min compared to 83 ± 11 mg/dL at 0 min (mean ± SD).[153]

D. The equine oral glucose absorption test has been paired with serial insulin measurements to identify insulin dysregulation rather than malabsorption. A modification of this is the so-called *oral sugar test*, for which glucose is substituted with 0.15–0.45 mL/kg body weight corn syrup (e.g., Karo Syrup Light). In addition to glucose, Karo Syrup Light contains the disaccharide maltose, which must be digested by intestinal maltase to form two glucose molecules prior to absorption.

IV. Disorders and conditions that alter oral glucose absorption curves (Table 15.11)

LACTOSE TOLERANCE TEST IN HORSES

I. Physiologic processes and introductory concepts: The lactose tolerance test is rarely used but could be used in foals to detect a lactase deficiency that typically is secondary to intestinal mucosal damage.[156] Lactase, which catalyzes the splitting of glucose and galactose from the disaccharide lactose, is typically present in the brush border of the small intestine in foals and young adult horses (< 3 yr old). Older horses lack the enzyme.[157] The fate of the glucose generated by the action of lactase is described in the prior section titled Glucose Absorption Test in Horses.

II. Analytical concepts (see Chapter 14, Glucose Concentration in Serum, Plasma, or Whole Blood, Section II)

III. Lactose tolerance test
- A. Basics of procedure: Lactose is administered via a stomach tube, and then multiple blood samples are collected to assess the intestinal mucosa's ability to digest lactose to glucose and galactose. Assuming normal glucose absorption and metabolism, the peak blood [glucose] should reflect the amount of lactase activity in the intestine.
- B. Procedure[158]
  1. Feed is withheld for 4 h.
  2. A blood sample is collected for a baseline (or 0-time) [glucose].
  3. Lactose monohydrate (1 g/kg body weight as a 20 % solution, weight/volume) is administered via stomach tube.
  4. Blood samples are collected at 30, 60, and 90 min after lactose is administered.
  5. Blood samples are processed appropriately, and glucose concentrations are measured in the serum or plasma samples.
- C. Criteria for adequate lactase activity
  1. [Glucose] is 150–250 % of baseline concentrations at 60 or 90 min.[155]
  2. A peak [glucose] is at least 35 mg/dL above the baseline [glucose].[158]

IV. Disorders and conditions that alter results of the oral lactose tolerance test
- A. Maldigestion caused by lactase deficiency (typically secondary)
  1. Viral enteritis (e.g., rotavirus)
  2. *Clostridium difficile* enterocolitis[159]
- B. Malabsorption of glucose because of disease of the small intestine
- C. Excessive cellular use of absorbed glucose

SUCROSE CONCENTRATION IN DOGS AND HORSES

I. Physiologic processes: Sucrose is a disaccharide that is split into glucose and fructose by sucrase in the proximal small intestine. Sucrose is typically not absorbed by either gastric mucosa (impermeable) or enteric mucosa (degraded before absorption can occur), so absorption indicates ulcerative or nonulcerative mucosal damage of the stomach or small intestine.

II. Analytical concepts
- A. Assays
  1. High-performance liquid chromatography with pulsed amperometric detection or mass spectrometry have been used to separate sucrose from other sugars and quantify it.[160–162]
  2. A spectrophotometric sucrose assay is available,[163] but reports of its use in domestic mammals were not found.
- B. Sample: urine, serum, or plasma

III. Sucrose absorption tests
- A. Basics of procedure: Details have varied, but reported procedures involve administering by nasogastric tube a solution of sucrose dissolved in water, and then collecting samples at fixed time points for sucrose measurement in urine or serum.
- B. In dogs, aspirin-induced gastropathy (including ulcers) was associated with increased urinary sucrose excretion, but focal ulcers induced by administration of ethanol into the gastric antrum were not.[160]
- C. Horses with gastric ulcers induced by intermittent feed deprivation had increased urine [sucrose], and urine [sucrose] was positively associated with ulcer severity score.[161] Urine

sucrose to osmolality ratios yielded similar results, suggesting that adjustment for differences in urine osmolality is not necessary. However, serum [sucrose] results have varied. In one study, horses with naturally occurring gastric ulcers had increased serum [sucrose], the peak [sucrose] being positively associated with ulcer severity,[162] but in another study using endoscopy as the gold standard to identify and grade gastric lesions, the diagnostic utility of serum [sucrose] was poor.[164]

OTHER METHODS OF EVALUATING DIGESTIVE OR ABSORPTIVE FUNCTIONS

I. Many other laboratory assays or methods are used to evaluate animals with potential exocrine pancreatic or intestinal diseases (Tables 15.12 and 15.13). Most of the assays listed in the tables are no longer or only rarely used, often because of unacceptably high rates of false-negative and false-positive results.

Table 15.12. Other laboratory methods that have been used to evaluate for EPI

Laboratory method	Abnormal result	Suggested pathologic state
Azocasein digestion	Feces failed to digest azocasein	↓ fecal proteolytic activity
Fecal fat excretion	↑ fecal fat in a 24-h sample	Maldigestion of ingested lipids
Fecal fat, microscopy	↑ Sudanophilic droplets in feces	Maldigestion of ingested lipids
Fecal starch, microscopy	↑ starch granules stained by Lugol's iodine	Maldigestion of ingested starch
Gelatin tube digestion	Feces failed to liquefy gelatin adequately	↓ fecal protease activity
Muscle fibers, microscopy	Undigested myocytes present	Maldigestion of dietary muscle (protein)
PABA[a]	↓ plasma [PABA] or ↓ urinary PABA excretion	↓ chymotrypsin released from pancreas
Plasma turbidity test	Absence of plasma turbidity after a lipid meal, but turbidity present after ingesting predigested lipids	↓ pancreatic lipase activity in intestine
Radiographic film digestion	Feces failed to clear gelatin from film	↓ fecal proteolytic activity
Starch digestion test	Flat glucose absorption curve	Maldigestion of starch or malabsorption of glucose

[a] PABA: N-benzoyl-L-tyrosyl-para-aminobenzoic acid (bentiromide)

Note: Increases in serum α-amylase and lipase activities can be indicators of active or recent pancreatic acinar cell damage. Serum α-amylase and lipase activities should not be used to detect EPI, because there are extrapancreatic sources of α-amylase and lipase that can keep serum activity WRI in EPI cases. Serum α-amylase and lipase activity can be decreased when an animal does not have EPI.

Table 15.13. Other laboratory methods that have been used to evaluate intestinal diseases

Laboratory method	Abnormal result	Suggested pathologic state
Bacterial culture (isolation)	Specific isolate (e.g., *Salmonella*)	Bacterial enteritis
Bacterial culture (quantitative)	> 10^5 CFU/mL of duodenal juice has been used	Small intestinal bacterial overgrowth, perhaps
Biopsy procedures (incision or excision)	Abnormal cell populations or abnormal tissue structure	Varies; typically inflammation or neoplasia
Direct fecal exam	Motile parasites	Giardiasis or amoebiasis
Fecal S100 protein tests (calprotectin, calgranulin)	↑ concentration	Inflammation: inflammatory bowel disease
Fecal flotation test	Parasitic ova, oocysts, or larvae	Parasitism
Fecal occult blood	Positive reaction for heme	Blood in alimentary tract if not false positive
Fecal sedimentation test	Fluke ova	Parasitism
Hydrogen breath test	Increased hydrogen production	Bacterial overgrowth or carbohydrate malabsorption
Plasma turbidity test with predigested fat	Absence of plasma turbidity	Malabsorption of digested lipids
Stained feces, microscopy	Acid-fast organisms	*Cryptosporidia* oocysts or *Mycobacterium* bacilli
	Giardia trophozoites	Giardiasis
	Histoplasma organisms	Histoplasmosis
	↑ leukocytes	Enteritis
	Neoplastic cells	Neoplasia
	Uniform bacilli	Bacterial overgrowth
Starch digestion test	Flat glucose absorption curve	Malabsorption of glucose
Unconjugated bile acids	↑ serum concentrations	↑ bacterial deconjugation of bile acids (intestinal dysbiosis)
Urine indican test	Positive for indican	↑ degradation of tryptophan by enteric bacteria
Urine nitrosonaphthol test	Positive nitrosonaphthol test	↑ bacterial degradation of tyrosine by enteric bacteria

Note: This is not a complete list. Other special assays (e.g., polymerase chain reaction, electron microscopy, and indirect fluorescent antibody testing) are used for specific organisms or pathologic states.
CFU, colony-forming units

A. The measurement of [TLI] has generally replaced other tests that were used to evaluate potential maldigestive disorders.
B. Most of the absorption tests have not been found to be clinically valuable. If malabsorption is suspected, histologic examination of biopsy samples is frequently used to establish a diagnosis so that appropriate therapy can be recommended or initiated.

II. Fecal assays used to detect intestinal parasitism are common in veterinary medicine, but discussion of the procedures is beyond the scope of this textbook. The reader is referred to diagnostic parasitology resources.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 16

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Kristen R. Friedrichs, Michael A. Scott, and Steven L. Stockham

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Table 16.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
CoA	Coenzyme A
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid, free fatty acid
HDL	High-density lipoprotein
IDL	Intermediate-density lipoprotein
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
NEFA	Nonesterified fatty acid, free fatty acid, fatty acid
RI	Reference interval
SI	Système international d'unités (International System of Units)
TG	Triglyceride (also called triacylglycerol)
TNF	Tumor necrosis factor
URL	Upper reference limit
VLDL	Very low-density lipoprotein

LIPIDS

I. A *lipid* is a compound that is soluble in organic solvents and relatively insoluble in H_2O. Of the lipid compounds in blood or other body fluids, cholesterol and TG are the most frequently assessed in domestic mammals, and thus are the focus of this chapter. Lipids found in mammals:[1]
   A. Sterols: cholesterol, cholesteryl esters, bile acids, steroid hormones, and vitamin D
   B. Glycerol esters: phosphoglycerides (phospholipids), monoglycerides, diglycerides, and TGs
   C. FAs: short-, medium-, and long-chain FAs, and prostaglandins (fatty-acid derivatives)
   D. Sphingosines: sphingomyelin (phospholipids), and glycosphingolipids
   E. Terpenes: vitamins A, E, and K

II. Lipids have many functions in the body but are particularly important as an energy source (TGs and FAs), as structural components of all cell membranes (phospholipids and cholesterol), and as substrates for hormones and second messengers.

OVERVIEW OF LIPOPROTEINS

I. Lipoprotein classification, content, and properties
   A. A basic understanding of lipoprotein composition and properties is needed to understand serum [TG] and [cholesterol] because all TG and most cholesterol molecules are transported in serum within lipoproteins. Formation of lipoproteins enables lipids to form small particles that can circulate in the aqueous plasma without coalescing into large lipid masses that would obstruct blood flow.
   B. Lipoproteins are classified either by their electrophoretic mobility or by their density relative to H_2O. Their properties depend on their composition (Figure 16.1 and Table 16.2).

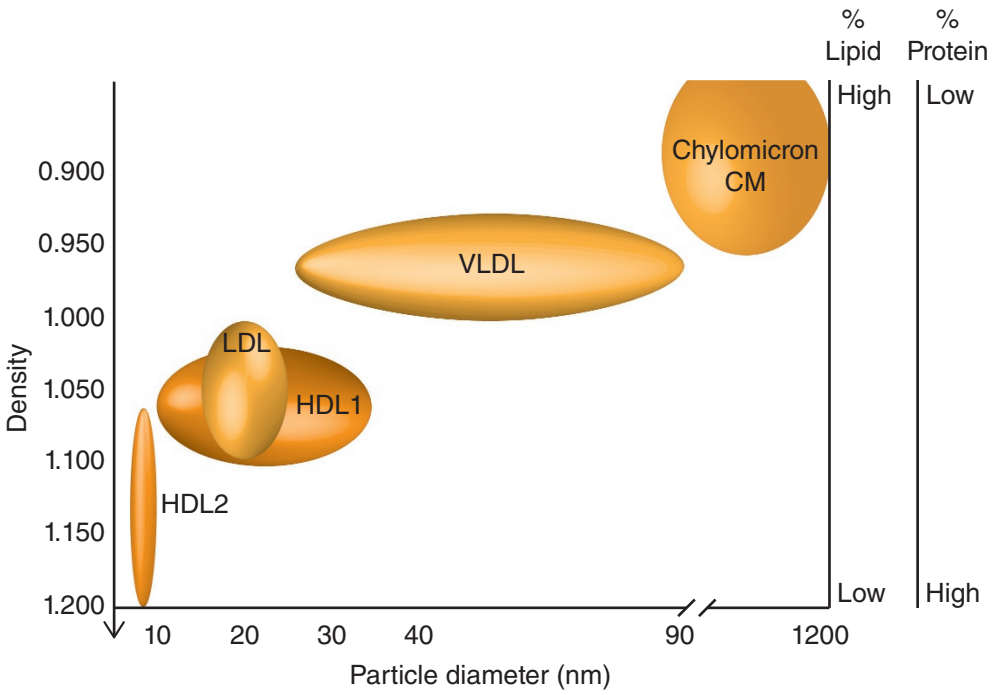


Figure 16.1. Graphic representation of selected lipoprotein characteristics in dogs.

- Density (*left vertical axis*) of lipoprotein particles, determined by relative quantities of lipid and protein (*right vertical axes*), is graphed relative to particle diameter (*horizontal axis*). The smaller, higher-density lipoproteins (HDL and LDL) are composed of greater proportions of protein relative to lipid, whereas larger, lower-density lipoproteins (VLDL and chylomicrons) consist of a greater proportion of lipid relative to protein. When their densities are less than those of serum or plasma, TG-rich chylomicrons rise to the surface upon standing. The relatively large size of chylomicrons and VLDL particles contributes to the turbidity (lactescence) they cause when at high concentrations. In this figure, the height and width limits of each type of lipoprotein indicates their reported variations in density and diameter, respectively.[2–4]
- The HDL2 distribution shown here is from Mahley's data;[2] the HDL2 label may be confusing because these HDL have characteristics that largely align with human HDL3. Consequently, some have referred to this fraction as HDL3 and consider dogs to lack HDL2. Others have recognized canine HDL2 and HDL3 in addition to HDL1 and defined them by human criteria such that canine HDL2 are HDL with diameters and densities of 9–12 nm and 1.063–1.100, respectively, and canine HDL3 are HDL with diameters of 5–9 nm and densities of 1.100–1.210, respectively (not shown).[3–5] With any of these approaches, the density profile of canine lipoproteins classified as HDL based on apolipoprotein content and electrophoretic mobility overlaps with that of canine LDL, even without considering HDL1, a negligible fraction in people. Densities of canine chylomicrons (< 0.960) and VLDL (0.930–1.006) are similar to those of people.[1]

1. The general composition of lipoproteins in domestic mammals is similar to the composition of human lipoproteins.
   a. The outside surface of lipoprotein particles contains phospholipids, nonesterified cholesterol molecules (so-called free cholesterol), apolipoproteins, and other proteins, many of which have functions not related directly to lipids. The hydrophilic aspects of the molecules are on the plasma surface; the hydrophobic aspects are adjacent

Table 16.2. Lipoprotein classification, content, and selected properties

Property	Lipoproteins				
	Chylomicron	VLDL	IDL	LDL	HDL
Predominant lipids	Dietary TG	Hepatic TG, phospholipid	Hepatic TG, cholesteryl esters, phospholipid	Cholesteryl esters, phospholipid	Phospholipid, cholesteryl esters
Apolipoproteins	A, B, C, E	B, C, E	B, C, E	B	A, C, D,[a] E
Electrophoretic migration	Origin	Pre-β	Between β and pre-β	β	α
Contribute to serum lactescence	Yes, floats with time	Yes	Maybe	No	No
Major site of formation	Small intestinal enterocytes	Hepatocytes	Plasma (from VLDL)	Plasma (from IDL and VLDL)	Hepatocytes, enterocytes
Major sites of degradation or transformation	Plasma, hepatocytes	Plasma	Plasma	Nonhepatic cells, hepatocytes, macrophages	Hepatocytes
Function	Transports dietary lipids to tissues (e.g., muscle, fat, mammary gland)	Transports hepatic lipid to tissues	VLDL remnant after TG transfer to tissues	Transports cholesterol from liver to tissues	Transports cholesterol from tissues to liver
Notable species differences					
Dogs and cats			May not exist		2 or 3 fractions in dogs;[b] 2 in cats
Cattle	Less TG and more phospholipid	Also Apo A; low liver synthesis	Also called LDL1; very little present	Comparatively low concentrations	3 fractions
Horses	Not detected in adults		Little or none	2–3 subfractions; Apo C and E too	1 fraction

Source: Modified from Remaley, 2012[1]

Apo = Apolipoprotein

[a] Apolipoprotein D is present mostly in HDL; very low amounts are present in LDL and VLDL.

[b] Some authors indicate three fractions while others indicate two.

to a core of mostly TG and cholesteryl ester molecules. FAs may be present in the core, but they also circulate independently of lipoproteins, bound to albumin.

b. *Apolipoproteins* (apoproteins) are the specific protein and polypeptide components of lipoproteins that provide structural integrity and regulate lipoprotein metabolism as ligands for lipoprotein receptors and as enzyme activators or inhibitors. They are separated into five major classes with subtypes: A (A-I, A-II, A-III, A-IV), B (B100, B48), C (CI, CII, CIII), D, and E.

c. Lipoprotein density increases with increased percentage of protein content, whereas the ratios of lipid to protein and TG to cholesterol decrease with increasing lipoprotein density (see Figure 16.1). Therefore, lipoprotein classes generally have the following relative densities: chylomicrons < VLDL < LDL < HDL. However, canine LDL and HDL densities overlap considerably (1.025–1.087) because of two factors:
   (1) The density range of canine LDL is large (greater than that of human LDL).
   (2) Canine lipoproteins defined as HDL1 by apolipoprotein content have a similar lipid-to-protein ratio as canine LDL. However, they contain very little TG and much more phospholipid and cholesteryl esters (see Table 16.2 for content and species variations). The cholesteryl esters appear to accumulate during reverse cholesterol transport (Figure 16.2).[3]

2. There are also significant variations in the relative amounts, content, and electrophoretic migration of lipoprotein fractions and subfractions among species.
   a. Dogs, cats, cattle, and horses have predominantly HDL cholesterol rather than LDL cholesterol. LDL cholesterol predominates in people. Predominance of HDL cholesterol is associated with decreased or absent cholesteryl ester transfer protein activity (transferring cholesterol from HDL to LDL and VLDL in exchange for TG) and likely contributes to a decreased susceptibility to atherogenesis.[6,7]
   b. Dromedary camels have a predominance of LDL, with lower concentrations of HDL and VLDL; this may be true of llamas and alpacas too.[8,9]
   c. Distinct subtypes of HDL are found in several species (Table 16.2).
   d. Identification of IDL varies by species and assessment method.

C. In veterinary medicine, TG and cholesterol are the routine screening tests to assess lipoproteins, whereas in human medicine, a routine lipoprotein profile also includes (i) measurement of HDL cholesterol ("good cholesterol" that transfers cholesterol from tissue to liver for catabolism and excretion), (ii) a calculation of LDL-cholesterol concentration ("bad cholesterol" that deposits cholesterol in tissue and vessel walls), and, sometimes, (iii) a calculation of VLDL-cholesterol concentration.

D. More complete lipoprotein analysis includes qualitative and quantitative lipoprotein subfraction analysis by various electrophoretic and density separations, as well as assessment of apolipoproteins by immunoassays or genetic tests. Such assays are used to assess lipoprotein metabolism in people but are rarely used in clinical veterinary medicine (see Other Assessments of Lipids).

II. Lipoprotein metabolism (Figures 16.2 and 16.3)

A. The three major processes that affect [lipoprotein] and thus [TG] and [cholesterol] are (i) synthesis of chylomicrons in enterocytes and VLDL in hepatocytes, (ii) LPL-catalyzed lipolysis on endothelial cell membranes, and (iii) hepatocyte uptake of lipoprotein remnants.
   1. Nascent (newly made) lipoproteins are produced in small intestinal enterocytes (chylomicrons and HDL) and in hepatocytes (VLDL and discoid HDL).
   2. Lipolysis of lipoproteins (chylomicrons, VLDL, and IDL) occurs on the luminal surface of capillary endothelial cells and is catalyzed by LPL.

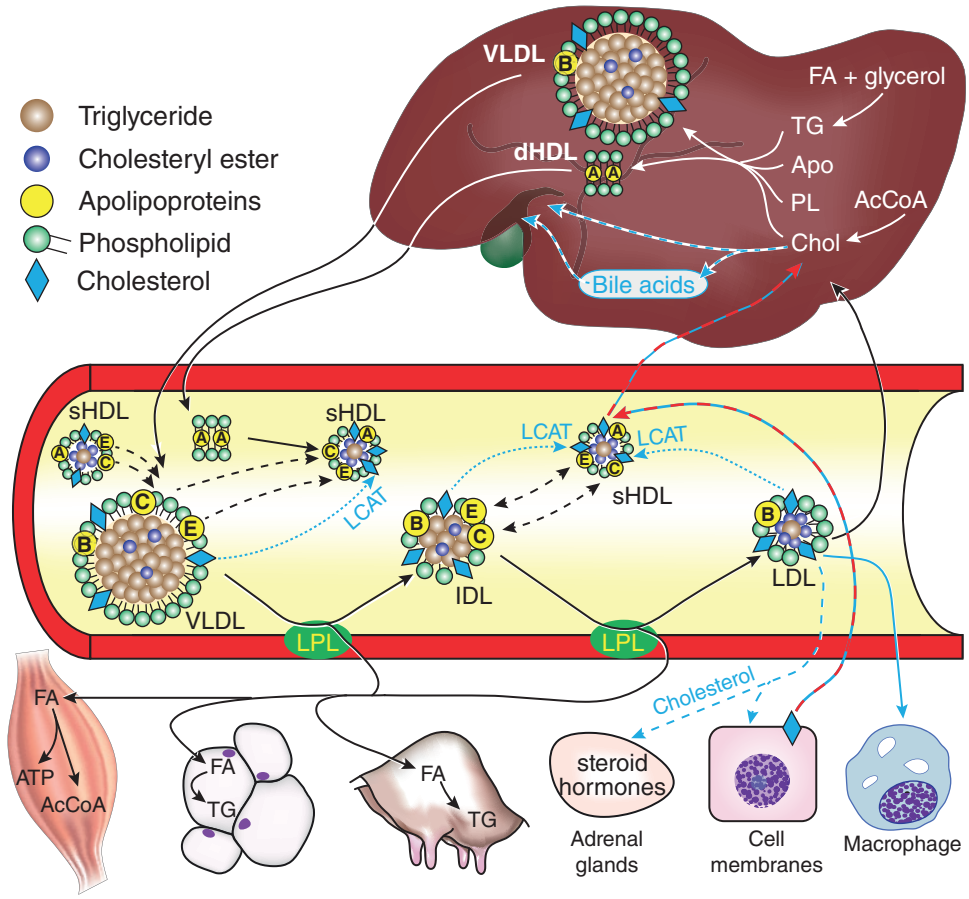


Figure 16.2. Basic physiologic processes: metabolism of endogenous lipids. Hepatocytes produce TG, phospholipids, apolipoproteins, and cholesteryl ester and assemble them into VLDL and dHDL. Cholesterol is derived from the diet (via chylomicron remnant uptake), de novo synthesis, or recycled remnant lipoproteins (e.g., LDL). Cholesterol that is not incorporated into lipoprotein is either metabolized to bile acids or directly eliminated in bile (*blue and white dashed arrow within liver*).

- TG-rich VLDL is the primary transporter of TG in the fasting state. VLDL assembled with ApoB-100 in hepatocytes is secreted into sinusoidal blood where it rapidly acquires apolipoproteins C and E from sHDL and subsequently enters the general circulation for delivery of FAs to tissues, including muscle, adipose tissue, and mammary gland.
  - When bound to ApoC-II on VLDL, LPL on endothelial cell membranes catalyzes the lipolysis of TG to generate FAs. As VLDL loses TG, it becomes denser to form IDL or VLDL remnants, which are removed by the liver (not shown). IDL undergoes further lipolysis to LDL.
  - LDL and LDL-cholesterol are delivered to many cells for maintenance of cell membranes or steroid hormone synthesis (*light blue dashed arrows*).
  - LDL clearance by hepatocytes involves the action of hepatic lipase and the binding of the cholesterol-rich remnant to an ApoB receptor on hepatocytes. LDL-cholesterol is also removed from circulation by macrophages in either receptor or nonreceptor-mediated processes (*light blue solid arrow*).
- dHDL consisting primarily of ApoA and phospholipid are produced extracellularly within the liver, and to a lesser degree in the intestine, and subsequently enter the general circulation.
  - dHDL accepts cholesterol, ApoC, and ApoE from circulating lipoproteins, which transform it into sHDL. Cholesterol is assimilated into sHDL by LCAT-catalyzed formation of cholesteryl ester from cholesterol (*light blue dotted arrows*), a process supported by ApoA-I. In this process, cholesterol ester moves to the core of

3. Lipoprotein remnants are cleared from plasma by hepatocytes, and, in the case of LDL, through endocytosis by many other types of cells as well.
4. In general, chylomicrons (from the intestine after eating) and VLDL (from the liver during fasting) transport TG (primarily composed of FAs) to tissues, while LDL delivers cholesterol to tissue, and HDL transports cholesterol from tissue to the liver for biliary excretion.

B. Apolipoproteins are critical for normal lipoprotein metabolism, influencing lipoprotein structure and solubility, acting as cofactors or inhibitors for enzymes involved in lipid metabolism, and acting as ligands for receptor-mediated cellular interactions including delivery of various lipid components to the appropriate tissues and cells.
1. The A class, from intestine and liver, is associated mostly with HDL. Apolipoprotein A-I is an activator of LCAT and a structural protein.
2. The B class, also from intestine and liver, is involved in synthesis and secretion of chylomicrons into lacteals and VLDL from hepatocytes to blood, and in the endocytosis of lipoprotein particles from VLDL, IDL, LDL, and chylomicron remnants. They are the only *nonexchangeable apolipoproteins*, being unable to transfer among lipoprotein particles.
3. The C class, from liver, is mostly in VLDL, HDL, and chylomicrons. Apolipoprotein C-II is a cofactor for LPL, while C-III inhibits it. In cattle, apolipoprotein C is primary associated with HDL because of the relatively low production of chylomicrons and VLDL in cattle.[10]
4. Circulating apolipoprotein D, an atypical apolipoprotein from many tissues, is mostly in HDL and is known to modulate LCAT activity.
5. The E class, from liver and macrophages, is in chylomicrons, VLDL, and remnant HDL where it mediates their uptake from plasma.

C. There are many other aspects of lipoprotein metabolism (e.g., mobilization and oxidation of lipids, apolipoprotein receptor regulation) that will not be considered here. For more detailed information about control and processes of lipoproteins, the reader is referred to textbooks or review articles.[3,5,9–14] A detailed understanding of lipoprotein metabolism is needed to understand coronary heart disease and some primary lipoprotein disorders. Because such disorders are uncommon in domestic mammals, lipoprotein information is limited here.

III. Lipases: Of the several lipases in the body, the major ones that pertain to lipid disorders are LPL, hepatic lipase (hepatic triglyceride lipase), and hormone-sensitive lipase. More information about lipases, including pancreatic lipase, is in Chapter 12.

A. Many extrahepatic cells, including adipocytes, myocytes, and macrophages, produce LPL. It migrates from these cells to the luminal surface of endothelial cells, where it catalyzes the hydrolysis of TG in chylomicrons, VLDL, and their remnants (IDL, LDL) to promote FA uptake. It also promotes uptake of lipoprotein remnants. Its activity is enhanced by

Figure 16.2. *continued*
sHDL, making it more spheroid and allowing incorporation of more free cholesterol into the membrane. sHDL also transports cholesterol from plasma membranes of tissues back to the liver for reutilization (in hepatocytes) or degradation (in hepatocytes or macrophages) in a process called *reverse cholesterol transport* (*dashed red/blue arrows*).
- sHDL serves as a repository of C and E apolipoproteins for other circulating lipoproteins.
- sHDL also acquires PL from LDL and IDL via HDL phospholipid transfer protein (not shown). As HDL particles accumulate cholesterol and PL in circulation, they become larger and acquire their spherical form.

Yellow circles containing A, B, C, and E are A, B, C, and E apolipoproteins (ApoA, ApoB, …); dHDL, discoid HDL; sHDL, spherical HDL

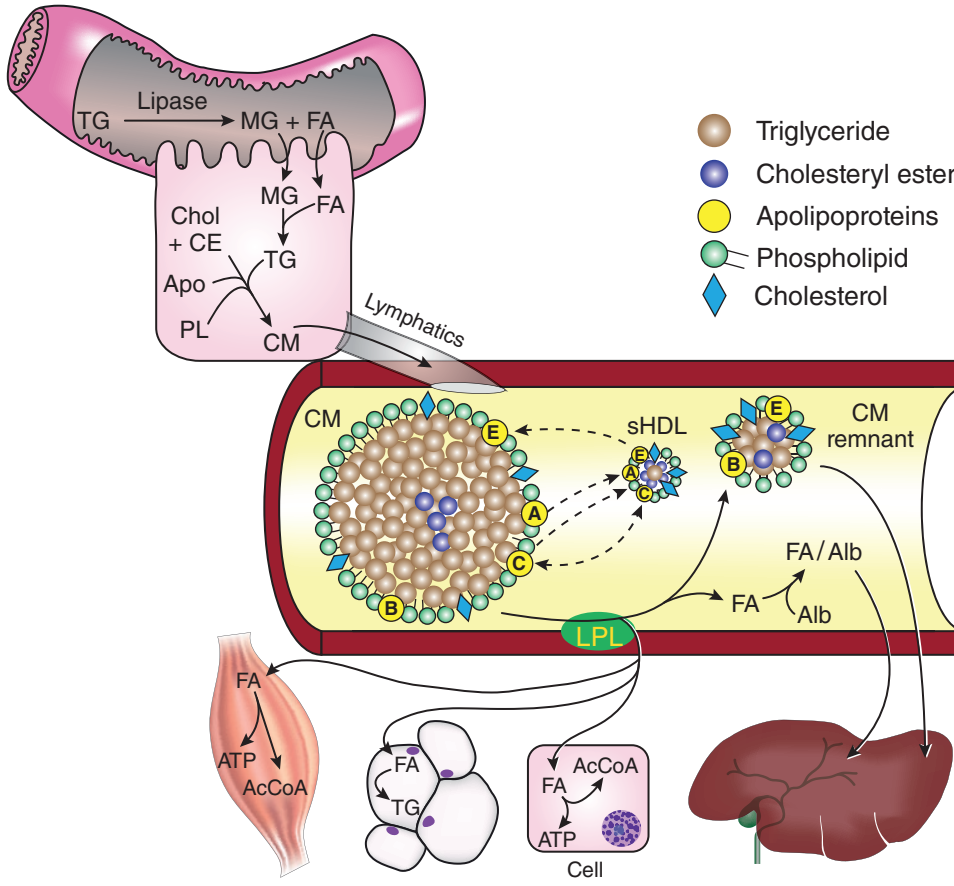


Figure 16.3. Basic physiologic processes: metabolism of exogenous or dietary lipids.

- Ingested TG in the presence of bile acids and pancreatic and intestinal lipases undergoes lipolysis to form MG and FAs. After absorption by enterocytes in the form of micelles, MG and FAs are reassembled into TG. Enterocytes produce CE, phospholipids, and apolipoproteins A and B-48 and then assemble the molecules into TG-rich lipoproteins called *chylomicrons*. Enterocytes also produce dHDL from intracellular cholesterol (not shown).
- The chylomicrons (and dHDL) exit enterocytes by exocytosis and enter lacteals through which they flow to larger lymphatic vessels and then enter blood via the thoracic duct, thus allowing postprandial lipids to be available to cells throughout the body prior to hepatic clearance. In blood, chylomicrons obtain C and E apolipoproteins from circulating sHDL.
- When bound to its cofactor ApoC-II on TG-rich lipoproteins (e.g., chylomicrons), LPL on endothelial cell membranes catalyzes the lipolysis of TG to generate FAs. The FAs enter myocytes to undergo oxidation to generate energy, enter adipocytes to be stored in TG, enter other cells for energy generation, or circulate bound to albumin and are cleared by the liver.
- After removal of most TG molecules and transfer of ApoC, ApoA, and PL to sHDL, the chylomicron remnants, which are high in cholesterol content, are cleared from plasma by hepatocytes in a process involving E apolipoproteins. Cholesterol from chylomicron remnants may be stored in the liver, used in the synthesis of nascent lipoproteins (VLDL and HDL) or bile acids, or eliminated in bile (Figure 16.2).

AcCoA, acetyl-CoA; Alb, albumin; ATP, adenosine 5′-triphosphate; CE, cholesteryl ester; Chol, cholesterol; CM, chylomicron; MG, monoglyceride; and PL, phospholipid; see Figure 16.2 for other abbreviations

thyroxine, heparin, and – depending on the organ – insulin, glucagon, and epinephrine. Insulin promotes LPL in adipose tissue but decreases LPL activity in muscle, whereas glucagon and epinephrine promote LPL activity in muscle. Therefore, adipose tissue LPL activity is increased postprandially to promote fat storage and myocyte LPL activity is increased during fasting, thus promoting increased FA uptake for generation of ATP.

B. Hepatic lipase is produced by hepatocytes, is present on endothelial cells of hepatic sinusoids, and hydrolyzes TG in LDL.

C. Hormone-sensitive lipase, located primarily within adipocytes, catalyzes the release of FAs from TG. Its activity is stimulated by glucocorticoid hormones, catecholamines, and glucagon; it is inhibited by insulin.

CHOLESTEROL CONCENTRATION

I. Physiologic processes

A. Cholesterol is often thought of in a negative light, but it is a critical component of cell membranes and a precursor for critical molecules: bile acids and steroid hormones including cortisol, aldosterone, progesterone, estrogen, testosterone, and vitamin D.

B. Cholesterol is absorbed from food into the body via specific jejunal carriers, but endogenous synthesis is a greater source.

C. Nearly all cholesterol in a fasting serum sample is within lipoproteins, and most was produced in hepatocytes. However, essentially all nucleated cells can synthesize cholesterol for intracellular use (e.g., for cell membranes). Particularly significant nonhepatic sources are gonads, adrenal glands, and intestinal mucosal cells, the last of which package cholesterol into chylomicrons.

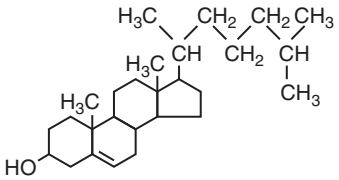
D. In the healthy state, cholesterol synthesis begins with acetyl-CoA and is a regulated process that depends on cellular [cholesterol]. The major rate-limiting enzyme is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase.

E. The potential toxic effects of unesterified (free) cholesterol, which can accumulate within cellular membranes, are prevented by cholesterol esterification involving two enzymes: plasma LCAT and cellular acylcholesterol acyltransferase. Esterification also increases the lipid-carrying capacity of lipoproteins by creating more-hydrophobic lipid molecules.

F. In general, LDL, derived from VLDL, delivers cholesterol produced in the liver to other cells in the body, and HDL scavenges excess cholesterol from cells throughout the body and delivers it to the liver.

G. Cholesteryl esters enter cells by receptor-mediated endocytosis of lipoproteins or lipoprotein fragments. They are delivered to lysosomes, where acid lipase promotes their hydrolysis. Cholesterol can then be released for use by the cell.

H. When cholesterol reaches hepatocytes via lipoprotein remnants, it may be reused for lipoprotein synthesis, excreted unchanged in bile, or degraded to bile acids.



Cholesterol

II. Analytical concepts

A. Terms and units

1. Serum or plasma [cholesterol] represents the total serum or plasma [cholesterol], including cholesterol and cholesteryl esters that are hydrolyzed during analysis. The reacting cholesterol molecules are within lipoproteins, typically in cholesterol-rich LDL and HDL.
2. Units: mg/dL × 0.02586 = mmol/L (SI unit)[15]

B. Sample
1. Serum and heparinized plasma are acceptable samples, with plasma yielding slightly lower results. Total [cholesterol] is stable at 4 °C for 3 d, and diagnostically significant changes have not occurred in samples frozen at −20 °C or −80 °C for as long as 2 yr.[16] Repeated thawing and refreezing is best avoided.[17]
2. Dogs and cats should be fasted overnight or for about 12–16 h to avoid postprandial hyperlipidemia, which may affect [cholesterol].

C. Methods
1. Most automated assays are enzymatic methods that hydrolyze cholesteryl esters and then use cholesterol oxidase to oxidize cholesterol and generate hydrogen peroxide, which then reacts with an indicator dye. Some assays use an O_2-sensing electrode to measure O_2 consumption.
2. High [bilirubin] and [ascorbic acid] may cause negative interference with some enzymatic assays.[1] Ascorbic oxidase may be included in an assay's reagents to remove the effect of ascorbic acid. Method-dependent drug interferences may occur.

III. Hypercholesterolemia
A. Increased serum [cholesterol] indicates there are increased concentrations of lipoproteins that contain cholesterol.
B. Disorders or conditions (Table 16.3)
C. Serum [cholesterol] may appear increased by up to about 60 % in kittens < 12 wk of age when compared to adult RIs.[18]
D. Serum [cholesterol] increased in atopic dogs treated twice daily for 2 wk with oclacitinib (Apoquel), a Janus kinase inhibitor; some dogs became hypercholesterolemic.[19]
E. The pathologic processes that cause hypercholesterolemia are described in a later section on lipoprotein disorders (see Hyperlipemia, Hyperlipidemia, and Hyperlipoproteinemia Disorders).

IV. Hypocholesterolemia
A. Based on the authors' collective clinical experience and reported findings in human patients, the reported causes of hypocholesterolemia in veterinary species are likely incomplete. Human conditions that seem likely to cause or contribute to hypocholesterolemia in domestic mammals are included in this section but are duly noted as human conditions.[20,21]
B. Disorders or conditions that cause hypocholesterolemia (Table 16.4)
1. Serum [cholesterol] may appear decreased by up to about 30 % in 20-wk-old weaned kittens when compared to adult RIs.[18]
2. Decreased production

Table 16.3. Disorders and conditions that cause hypercholesterolemia[a]

Physiologic state			
Kittens < 12 wk of age			
Multiple mechanisms	↑ lipoprotein production[b]	↓ intravascular lipolysis	↓ hepatic uptake
Hypothyroidism*		+	+
Hyperadrenocorticism*	+	+	+
Glucocorticoid-associated in cats	+	+	+
Diabetes mellitus*	+	+	
Cholestasis (obstructive)*	+		+
Nephrotic syndrome or protein-losing nephropathy*	+	+	+
Other or unknown mechanisms			
Pancreatitis, acute			
Idiopathic hyperlipidemia of miniature schnauzers			
Idiopathic hyperlipidemia of Shetland sheepdogs			
Hypercholesterolemia in briard dogs			
Hypercholesterolemia in rough collies			
Equine hyperlipemia (hyperlipidemia syndrome), mostly ponies			
Transient hyperlipidemia in preweaned kittens			
Oclacitinib (Janus kinase inhibitor) administration			

* A relatively common disorder, condition, or mechanism

[a] See the text for evidence or theories of pathogeneses.

[b] ↑ lipoprotein production = increased production of cholesterol-containing lipoproteins; ↓ intravascular lipolysis = decreased lipolysis or intravascular processing of lipoproteins; ↓ hepatic uptake = decreased clearance of lipoproteins or lipoprotein-remnants by hepatocytes

Table 16.4. Disorders and conditions that cause hypocholesterolemia[a]

Physiologic state
Weaned kittens (20 wk of age)*
Decreased production
Liver-related disorders
Hepatic atrophy due to portosystemic vascular anomaly (shunt)*
Hepatic failure
Hepatic disease due to aflatoxicosis
Hepatic lipidosis in cattle and cats
Infectious and noninfectious inflammatory disease
Multiple mechanisms[a]
Protein-losing enteropathy*
Critical patients
Other or unknown mechanisms
Hypoadrenocorticism including selective glucocorticoid deficiency
Neoplastic states (feline myeloma and lymphoma, canine hemophagocytic syndrome, other)
Congenital cholesterol deficiency in Holstein cattle with apolipoprotein B gene mutation

* A relatively common disorder, condition, or mechanism

[a] See the text for evidence of pathogeneses or for theories.

a. Liver-related disorders
   (1) Portosystemic vascular anomaly (also called portosystemic shunt): It is reported that about 60–70 % of dogs and cats with portosystemic vascular anomalies have hypocholesterolemia.[22] Pathogeneses proposed for the hypocholesterolemia: (i) decreased cholesterol production because of decreased functional hepatic mass, and (ii) inhibition of cholesterol synthesis by bile acids. Dogs with hepatic microvascular dysplasia, a disease with a similar presentation, typically have a [cholesterol] within RI.[23]
   (2) Hepatic failure (decreased hepatic functional mass): Although supporting published data are lacking for domestic mammals, acute or chronic hepatic failure may cause hypocholesterolemia, as is commonly stated; this may be because of decreased cholesterol synthesis.[24]
   (3) Aflatoxicosis: Hypocholesterolemia occurred in a majority of dogs with aflatoxin hepatotoxicosis. Impaired synthesis of cholesterol and certain other proteins, specifically by aflatoxin rather than because of generalized hepatocellular damage, was the speculated cause.[25]
   (4) Hepatic lipidosis in dairy cattle and cats: Postpartum dairy cows with moderate to severe hepatic lipidosis may be hypocholesterolemic. Decreased hepatic synthetic function is suspected to be the cause.[26] In one study, hypocholestrolemia was present in 26 % of the 65 tested cats with hepatic lipidosis, and it was associated with mortality.[27]

b. Infectious and noninfectious inflammatory conditions are commonly associated with hypocholesterolemia in people.[20,21] This includes sepsis and chronic immune-mediated conditions. Inflammatory cytokines (interleukin-1β, interleukin-6, and TNFα) are suspected to affect plasma cholesterol concentrations, perhaps by decreasing apolipoprotein synthesis. In a study focused on [cholesterol] in septic dogs, only 3 of 51 septic dogs had hypocholesterolemia at the time of diagnosis, while 8 were hypercholesterolemic.[28]

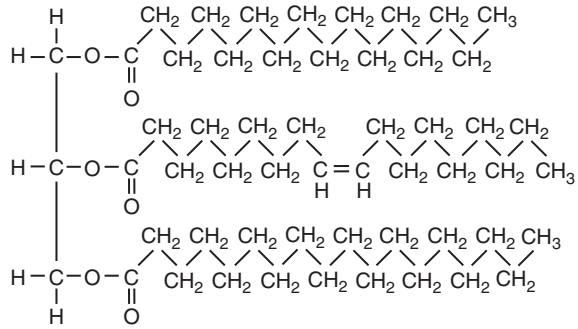
3. Protein-losing enteropathy (especially because of intestinal lymphangiectasia):[29–31] The pathogenesis of hypocholesterolemia may include decreased lipoprotein production because of the catabolic state or, in the case of lymphangiectasia, intestinal loss of lipids that are produced by enterocytes.
4. Critically ill, hospitalized human patients with a variety of conditions are commonly hypocholesterolemic, the degree of which has prognostic value, and the resolution of which reflects recovery.[20,21]
   a. Hypocholesterolemia is likely multifactorial in many of these patients, being related to inflammation (see Hypocholesterolemia, Section B.2.b), decreased food (and therefore cholesterol) intake, hepatic dysfunction, cholesterol loss through blood loss or purulent discharges (cell membranes), hemodilution from fluid administration, or increased utilization and incorporation into cells because of tissue repair, regenerative anemias, or proliferating neoplasms.
   b. In a study of 398 critically ill dogs with a variety of disorders, including some associated with hypercholesterolemia, hypocholesterolemia occurred in about 20 % of nonsurvivors and 12 % of survivors (14 % overall).[32]
      (1) All 10 critically ill dogs with concurrent hypertriglyceridemia and hypocholesterolemia were nonsurvivors.
      (2) The reduced cholesteryl ester transfer protein activity in dogs compared to people may promote maintenance of serum cholesterol concentrations within HDL despite reduced cholesterol production in inflammatory states.

5. Hypoadrenocorticism including selective glucocorticoid deficiency
   a. In one study involving 17 dogs with hypoadrenocorticism, 13 had hypocholesterolemia.[33] In another study involving 201 dogs, 14 were hypocholesterolemic, but 29 had hypercholesterolemia.[34] In a study of 40 dogs with selective glucocorticoid deficiency, 76 % were hypocholesterolemic.[35]
   b. Hypocholesterolemia is reportedly more common in dogs with selective glucocorticoid deficiency than in dogs with combined mineralocorticoid and glucocorticoid deficiencies.[36]
   c. Cortisol influences lipoprotein metabolism in dogs with hyperadrenocorticism (see Hyperlipemia, Hyperlipidemia, and Hyperlipoproteinemia Disorders, Section V. A.2), but how hypocortisolemia is associated with hypocholesterolemia is not established.
6. Certain neoplastic diseases
   a. Hypocholesterolemia was reported in 11 of 16 cats with plasma cell myeloma,[37] 11 of 46 cats with nasal lymphoma,[38] and 11 of 16 dogs diagnosed with hemophagocytic histiocytic sarcoma.[39] Of 285 dogs with moderate to marked hypocholesterolemia, a neoplastic process was diagnosed in 58 dogs (~20 %), and half of those dogs had lymphoma, but the type of lymphoma and involved organs were not reported.[40]
   b. Contributors to hypocholesterolemia associated with these neoplasms may include increased lipid metabolism by neoplastic cells, increased cholesterol utilization, and decreased cholesterol production associated with high oncotic pressure, inflammatory cytokines, or hyporexia.
   c. In people, hypocholesterolemia has been associated with malignancies including multiple myeloma and acute myeloid leukemias; increased LDL receptor activity and LDL metabolism by neoplastic cells may play roles.[20]
7. Hereditary hypocholesterolemia in Holstein cattle (commonly referred to as cholesterol deficiency)[41–43]
   a. This genetic disorder is caused by a loss-of-function insertional mutation in the apolipoprotein B gene (*APOB*), which results in impaired lipoprotein production and cholesterol transport. The mutation was traced back to Maughlin Storm, a Canadian Holstein sire used for artificial insemination. The disorder was first described as autosomal dominant but is now thought to have incomplete dominant inheritance with incomplete penetrance.
   b. There are two isoforms of apolipoprotein B. The larger isoform is synthesized in hepatocytes where it is important for VLDL assembly. It also provides structural integrity to VLDL, IDL, and LDL, and attaches to cell membrane receptors to facilitate lipid transport into cells. A smaller, truncated, isoform is synthesized in small intestinal enterocytes where it is important for assembly of chylomicrons and subsequent transport of absorbed lipids from enterocytes to lymphatic vessels and then blood. This truncated isoform lacks the ligand for LDL receptors.
   c. Homozygous cattle (mostly calves) typically have marked hypocholesterolemia, marked hypotriglyceridemia, impaired growth, poor body condition, and chronic intermittent diarrhea with steatorrhea; they die prematurely, often by 6 mo. Histologic changes support failure to export lipids absorbed from the intestinal lumen, which is consistent with decreased functional apolipoprotein B and impaired chylomicron formation.
   d. Heterozygous cattle (carriers) typically have reduced [cholesterol] and [TG] in the absence of clinical signs, but clinically affected heterozygotes have been reported.

TRIGLYCERIDE (TG) (TRIACYLGLYCEROL) CONCENTRATION

I. Physiologic processes
   A. TG provides a highly concentrated form of energy.
   B. In general, lipoproteins transport TG from intestine and liver to muscle for energy or to adipose tissue for efficient anhydrous storage.
   C. Nearly all TG in a fasting serum sample is within lipoproteins that were produced in hepatocytes. Much of the TG in a postprandial sample is in chylomicrons that were assembled in enterocytes from dietary lipids.
   D. TG synthesis begins with FAs (short, medium, or long chain) and glycerol from carbohydrate metabolism. TG synthesis occurs in hepatocytes and enterocytes.
   E. When lipoproteins containing apolipoprotein C-II bind to LPL on endothelial cells, FAs are cleaved from TG molecules and enter cells (e.g., adipocytes and myocytes). With repeated processing, the lipoproteins become TG depleted and thus serum [TG] decreases.
   F. Intravenous heparin promotes the release of LPL from endothelial cells and thus an increased plasma LPL activity.[1] Heparin also promotes release of hepatic lipase from the sinusoidal endothelial cells to plasma.[4,44] Because of its actions, heparin is sometimes called *lipemia clearing factor*.

II. Analytical concepts
   A. Terms and units
      1. *Triglyceride* is the more common term for a group of lipids that contain three FAs attached to a glycerol backbone (the illustrated molecule contains palmitic, oleic, and stearic acids). The more correct term for the molecule is *triacylglycerol*, but it is not used as frequently in medical communication.



Triglyceride (triacylglycerol) example

      2. Serum or plasma [TG] represents the total serum or plasma [TG]; the reacting TG molecules are mostly within TG-rich lipoproteins (i.e., chylomicrons, VLDL, and IDL). TG molecules are a highly variable population of molecules because of the varied FAs that are attached to the glycerol backbone.
      3. Units: mg/dL × 0.01129 = mmol/L (SI unit)[15]
   B. Sample
      1. Serum or heparinized plasma are acceptable samples, although plasma yields slightly lower results. Total [TG] is stable at 4 °C for 5–7 d, at −20 °C for 3 mo, and at −70 °C for years. Repeated thawing and refreezing are best avoided.[17]

2. Vacuum tubes with glycerin-coated stoppers have not been recommended for TG analysis because glycerin (glycerol) may be detected in assays that measure glycerol released from TG. However, in a pilot study with the Vitros TG assay, exposure of serum to glycerin-coated stoppers did not increase the measured [TG] (authors' unpublished data).
3. Increased concentrations of chylomicrons, VLDL, and possibly IDL may cause lactescence (turbid serum) and can interfere with a variety of assays.
   a. These lipoproteins may alter light transmission in spectrophotometric, nephelometric, and immunoturbidometric assays for a variety of analytes.
   b. They interfere with indirect potentiometry for electrolyte measurement.
   c. When a lipid extraction step is used to assess lipophilic analytes (e.g., steroid hormones, phenobarbital), partitioning into the nonpolar lipid fraction of the sample may yield falsely decreased values.
   d. Depending on reagents and detection methods, interference may result in falsely increased or falsely decreased results.
4. Laboratory personnel may reduce or eliminate chylomicrons and VLDL from serum samples by addition of clearing agents or by high-speed centrifugation (g ≥ tens of thousands), including ultracentrifugation (g = hundreds of thousands),[45] prior to chemical analysis. Ultracentrifugation most effectively separates lipemia-inducing lipoproteins from serum samples, whereas high-speed centrifuges that exert forces below those of ultracentrifuges are less effective, particularly for VLDL. When the serum is cleared of the TG-rich lipoproteins, the [TG] in the sample will be lowered. Harvesting serum below a superficial "cream" layer after routine centrifugation may also falsely decrease serum [TG].
5. Dogs and cats should be fasted overnight or for about 12–16 h to avoid physiologic postprandial hyperlipidemia. Prolonged postprandial hyperlipidemia indicates the presence of a pathologic state.

C. Methods
1. In most automated assays, the initial reaction involves a lipase that liberates glycerol from TG. The liberated glycerol is then measured in coupled reactions that result in products that can be detected by spectrophotometry or, for point-of-care strip methods, by reflectance photometry.
2. High [free glycerol] in serum (hyperglycerolemia) would produce falsely elevated [TG], but such conditions (glycerol kinase defects) are rare in people and have not been diagnosed in domestic mammals.
3. The [TG] in a grossly lipemic sample may exceed an assay's analytical range, and thus the sample may need to be diluted prior to analysis.
4. Point-of-care instruments for measuring [TG] in whole blood samples have been evaluated in cats, dogs, and horses; they appear useful for detecting moderate hypertriglyceridemia but may lack the dynamic range to accurately measure marked hypertriglyceridemia.[46–48]

III. Hypertriglyceridemia
A. Increased serum [TG] indicates there are increased concentrations of lipoproteins that contain TG.
B. Disorders or conditions (Table 16.5)
C. Physiologic hypertriglyceridemia: Hypertriglyceridemia occurs in foals after colostrum ingestion, and [TG] may remain increased compared to adult RIs for 2 wk or longer.[49]

Table 16.5. Disorders and conditions that cause hypertriglyceridemia[a]			
Physiologic state			
Kittens < 12 wk of age			
Postcolostral foals and possibly calves			
Increased production of lipoproteins			
Postprandial hyperlipidemia*			
Equine hyperlipemia (hyperlipidemia syndrome)*			
Negative energy balance in equids (hyperlipidemia without hyperlipemia)*			
Decreased lipolysis or intravascular processing of lipoproteins			
LPL deficiency (rare)			
Multiple mechanisms[b]	↑ lipoprotein production	↓ intravascular lipolysis	↓ hepatic uptake
Canine hypothyroidism		+	+
Hyperadrenocorticism	+	+	+
Diabetes mellitus*	+	+	
Nephrotic syndrome or protein-losing nephropathy*	+	+	+
Hepatic lipidosis in cats	+	+	
Endotoxemia, Gram-negative sepsis*	+		+
High catecholamine states in llamas and alpacas*	+	+	+
Other or unknown mechanisms			
Equine metabolic syndrome*			
Equine pituitary pars intermedia dysfunction*			
Idiopathic hyperlipidemia of miniature schnauzers*			
Idiopathic hyperlipidemia of Shetland sheepdogs			
Pancreatitis, acute (may be a cause or an effect)*			
Phenobarbital-associated hyperlipidemia			
Transient hyperlipidemia in preweaned kittens			
Propofol administration (soybean oil TG)			

* A relatively common disorder, condition, or mechanism

[a] See the text for evidence of pathogeneses or for theories.

[b] ↑ lipoprotein production = increased production of TG-containing lipoproteins; ↓ intravascular lipolysis = decreased lipolysis or intravascular processing of lipoproteins; ↓ hepatic uptake = decreased clearance of lipoproteins or lipoprotein-remnants by hepatocytes

Transient post-colostrum increases also have been reported in calves. Serum [TG] might be greater in kittens < 12 wk of age when compared to adult RIs.[18]

D. Pharmacologic hypertriglyceridemia

1. Propofol administration increased serum [TG] after bolus induction and to a greater degree after constant-rate infusion for 90 min to bitches undergoing ovariohysterectomy; [TG] exceeded the URL in some dogs.[50] Propofol is delivered in a lipid emulsion of soybean oil that contains TG and glycerol. Infused oil droplets acquire apolipoproteins in circulation and are thought to be metabolized like chylomicrons.[51]

2. Intravenous lipid therapy for lipophilic toxicants may cause transient hypertriglyceridemia as was reported in a cat with pyrethrin toxicity.[52] The cat had marked hypertriglyceridemia (10 × URL) and corneal lipidosis 36 h after infusion of lipid. Corneal cloudiness resolved by about day 3, but mild hypertriglyceridemia (3 × URL) was present on day 10, so an underlying dyslipidemia cannot be excluded as a contributing factor.

E. The pathologic processes that cause hypertriglyceridemia are described in a later section (see Hyperlipemia, Hyperlipidemia, and Hyperlipoproteinemia Disorders).

IV. Hypotriglyceridemia

A. Significant pathologic states with hypotriglyceridemia are not recognized in dogs, cats, horses, llamas, or alpacas.

B. The [TG] in a group of dogs with untreated exocrine pancreatic insufficiency was reportedly lower than the [TG] in a group of healthy dogs, but hypotriglyceridemia may not have been present.[53]

C. Hypotriglyceridemia is described in Holstein cattle with mutations in the *APOB* gene (see Cholesterol Concentration, Section IV.B.7).[41]

D. Some cattle that are in a negative energy balance have low [TG] concurrent with increased [NEFA] and [β-hydroxybutyrate].[54] This occurs in the postpartum period when low adipose-tissue LPL activity is thought to facilitate uptake of circulating TG by the mammary gland for synthesis of milk lipids.[55]

E. Lactation in cats and dogs may promote lower [TG], but not necessarily hypotriglyceridemia.[56,57]

NONESTERIFIED FATTY ACID (NEFA) CONCENTRATION

I. Physiologic processes (Figure 16.4)[58]

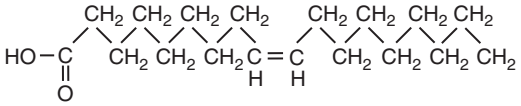
A. FAs are major contributors to energy metabolism in post-absorptive and fasting states.[58] They may be used for generation of energy (ATP), synthesis of amino acids, synthesis of milk lipids, or energy storage as TG in adipocytes.

B. Ruminants synthesize relatively few FAs in the liver, most being produced in adipose tissue and lactating mammary glands from acetate generated by microbial digestion in the rumen.[59,60] They also generate less TG and VLDL in hepatocytes, and export less hepatic VLDL for delivery of FAs to other tissues.

II. Analytical concepts

A. Terms and units

1. FAs are carboxylic acids with short, intermediate, or long carbon chains that may be saturated or unsaturated, the latter having carbon-carbon double bonds. The provided example, oleic acid, is an 18-carbon (long-chain), monounsaturated (1 double bond) FA.



A fatty acid (oleic acid)

2. FAs are commonly called free fatty acids because they are outside of lipoprotein particles, but most circulate bound to albumin and are not free in serum or plasma. The

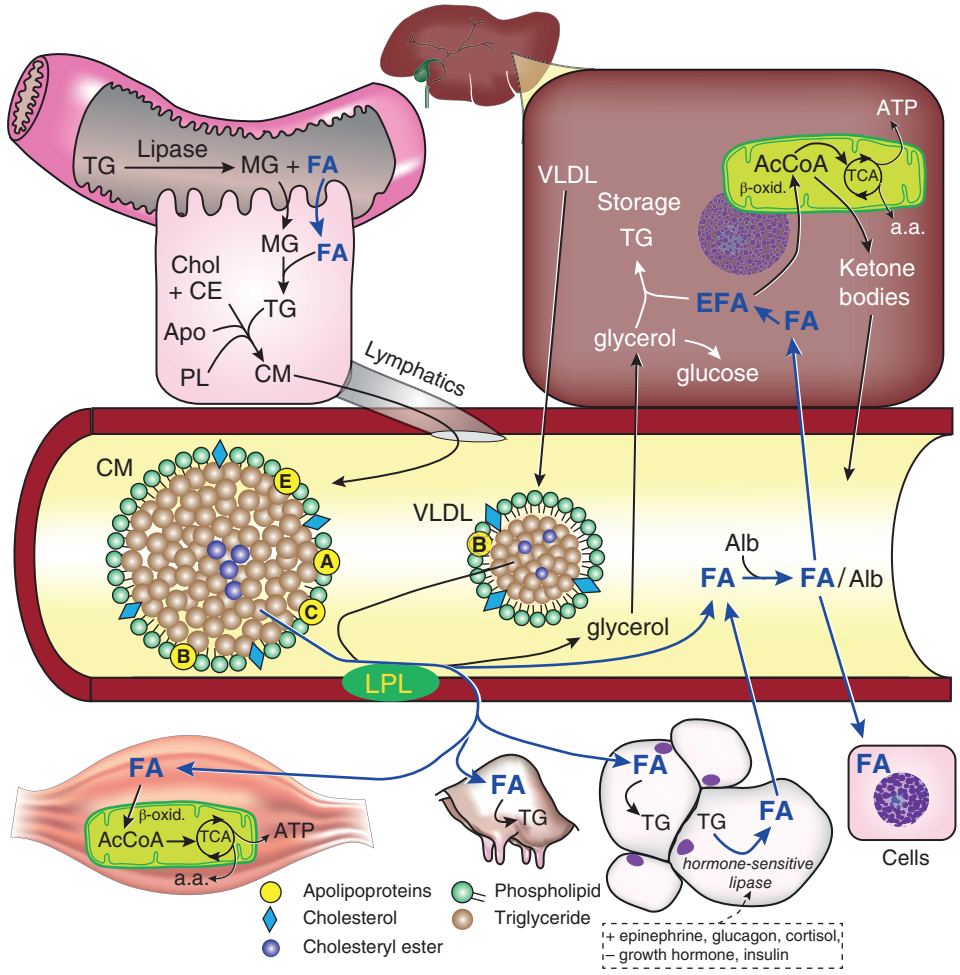


Figure 16.4. Basic physiologic processes: metabolism of FAs (NEFAs).

- FAs generated by LPL: As illustrated here and in Figures 16.2 and 16.3, FAs are formed in adipose tissue, muscle, and mammary gland by endothelial-associated, LPL-mediated hydrolysis of TG in chylomicrons and VLDL, which are produced by enterocytes and hepatocytes, respectively. FAs may be used locally or circulate to other tissues.
- FAs generated by hormone-sensitive lipase: During fasting and negative energy balance, hormone-sensitive lipase generates FAs through lipolysis of TG stored in adipocytes. Hormone-sensitive lipase activity is promoted by epinephrine, glucagon, and cortisol and inhibited by growth hormone and insulin, the latter limiting mobilization of energy stores postprandially. Released FAs enter circulation and are bound to albumin for delivery to liver and other tissues.
- Fate of FAs and glycerol: Circulating or locally generated FAs are passively taken up by myocytes, hepatocytes, or other cells and conjugated to CoA, which undergoes β-oxidation in mitochondria to form AcCoA; AcCoA enters the TCA for generation of energy (ATP) or conversion into amino acids. In the liver, AcCoA may also be used for ketogenesis. FAs are also incorporated into TG and subsequently VLDL in hepatocytes, into milk fat in mammary epithelium, and into storage TG in adipocytes. Glycerol generated along with FAs during lipolysis enters hepatocytes and is converted to glyceraldehyde 3-phosphate, an intermediate in glycolysis and gluconeogenesis.

a.a., amino acids; AcCoA, acetyl-CoA; Alb, albumin; ATP, adenosine 5′-triphosphate; CE, cholesteryl ester; Chol, cholesterol; CM, chylomicrons; MG, monoglyceride; PL, phospholipid; TCA, tricarboxylic acid cycle

International Union of Pure and Applied Chemistry (IUPAC) recommended term is *fatty acids*. NEFA is the commonly used test name in veterinary medicine.

3. Units: mEq/L × 1 = mmol/L

B. Sample

1. Plasma from EDTA- or heparin-anticoagulated blood or serum may be used for analysis of fresh samples.[61]
2. Serum or plasma should be separated from cells as soon as possible and be frozen or maintained at 4 °C before testing. Refrigerated samples are best tested within 24 h. Frozen samples should yield valid results for at least 1 mo. Values may be falsely increased if serum separator tubes (Corvac™ tube) are used, if samples are not kept cool, or if testing of samples kept at 4 °C is delayed beyond 24 h. When stored at 24 °C for 24, 48, or 72 h, [NEFA] increased in serum and plasma, with the greatest change occurring in heparinized plasma.[61]
3. Significant false increases also occur with moderate to severe hemolysis.[62]
4. To monitor lipid mobilization in prepartum cattle, samples should be collected shortly before feeding time (to reflect the peak lipolytic state) and 3–14 d before calving. They can be processed and stored frozen until calving occurs, at which time it will be known whether the samples were collected in the desired 3–14 d precalving window.
5. For postpartum assessment of bovine energy balance, sample collection 3–14 d after calving has been recommended.[63]

C. Methods

1. [FAs] may be measured in automated systems with an enzymatic colorimetric assay in which FAs are enzymatically acylated and then oxidized to hydrogen peroxide linked to a reaction that generates a colored product.
   a. This assay detects medium- to long-chain (6–26 carbon) FAs.
   b. Volatile FAs (e.g., propionic acid and butyric acid) are not measured.
2. Enzymatic methods replace more time-consuming nonenzymatic methods that rely on extraction of FAs by organic solvents after converting them to copper salts.

III. Increased NEFA concentration

A. [NEFA] increases as a response to a negative energy balance, including that caused by decreased food/feed intake. Excessive increases often reflect an underlying cause for decreased intake and may lead to further pathologic changes, such as hepatic lipidosis (see Hyperlipemia, Hyperlipidemia, and Hyperlipoproteinemia Disorders, Sections V.C and D). Mild increases can also occur postprandially.

B. Ruminants [63]

1. Increases occur with increased fat mobilization in response to a negative energy balance from any cause.
2. [NEFA] is used to monitor dairy cows in the 2- to 3-wk transition period prior to and following calving, when there are high energy demands for the fetus and for milk production (see Herd-based Testing for Cattle in Chapter 1).[64]
   a. When a certain proportion of prepartum cows in a dairy herd have values above a decision threshold, it is evidence that too many cows are in a negative energy balance, and management changes are needed.
   b. Excessively increased prepartum or postpartum [NEFA] in individual cows is associated with more postparturient ketosis, fatty liver, metritis/retained placentas,

and displaced abomasums, as well as decreased milk production and reproductive performance.

C. Alpacas and llamas: Mild increases in [NEFA] are expected during fasting; however, moderate to marked increases in [NEFA] in underfed alpacas and llamas enhance the risk of hepatic lipidosis.[65]

D. Dogs and cats: [NEFA] may be increased above baseline or occasionally above the URL after endurance exercise[66] or with food deprivation, overfeeding and obesity,[67] diabetes mellitus,[68] and hepatic lipidosis.[69]

E. Horses: Similar states promote an increase in [NEFA] in horses. The increases occurring with decreased food intake are commonly reflected by hyperbilirubinemia in horses that are off feed for any reason (see Chapter 13, Bilirubin Concentration, Section III.E.1).[70]

HYPERLIPEMIA, HYPERLIPIDEMIA, AND HYPERLIPOPROTEINEMIA DISORDERS

I. Terms

A. Hyperlipemia, hyperlipidemia, and hyperlipoproteinemia are nearly synonyms.

1. *Hyperlipemia* and *hyperlipidemia* refer to an increased lipid concentration in blood, as evidenced by hyperlipoproteinemia, hypercholesterolemia, hypertriglyceridemia, or increased [NEFA].
2. *Hyperlipoproteinemia* is an increased concentration of one or more lipoproteins in blood. It is the preferred term when a measured concentration of a lipoprotein (e.g., LDL or HDL) is increased.
3. The terms are sometimes used to identify clinical disorders (e.g., equine hyperlipidemia or equine hyperlipoproteinemia).

B. *Lipemia* (also lipidemia), by most dictionary definitions, is a synonym for both hyperlipidemia and hyperlipoproteinemia. In routine clinical use, lipemia is usually defined as the turbid or opaque appearance of serum or plasma as seen by the naked eye (Figure 16.5). The lactescence is primarily caused by increased concentrations of the large, TG-rich lipoprotein particles (i.e., chylomicrons and VLDL). If lipemia is present, moderate or marked hypertriglyceridemia is expected, but even marked hypercholesterolemia may occur without lipemia, and lipemia may be absent with marked hypertriglyceridemia in horses.[71] Lipoprotein factors other than their TG content (e.g., size) affect lactescence.

C. *Dyslipidemia* refers to any abnormality in the amounts or types of lipids in the blood, including hyperlipidemia, abnormal proportions of lipoproteins, and abnormal lipoproteins.

II. Classification of hyperlipoproteinemia conditions and disorders

A. *Physiologic* (postprandial) *hyperlipidemia* is the hyperlipidemia that occurs after ingestion of a meal containing TG.

B. *Primary* (or familial) *hyperlipoproteinemia* disorders are caused by congenital defects in lipoprotein metabolism; they are relatively uncommon in domestic mammals. The defects involve any of three major processes:[1]

1. Increased production of lipoproteins by hepatocytes
2. Defective intravascular processing of lipoproteins, including defective lipolysis
3. Defective cellular uptake of lipoproteins or lipoprotein remnants

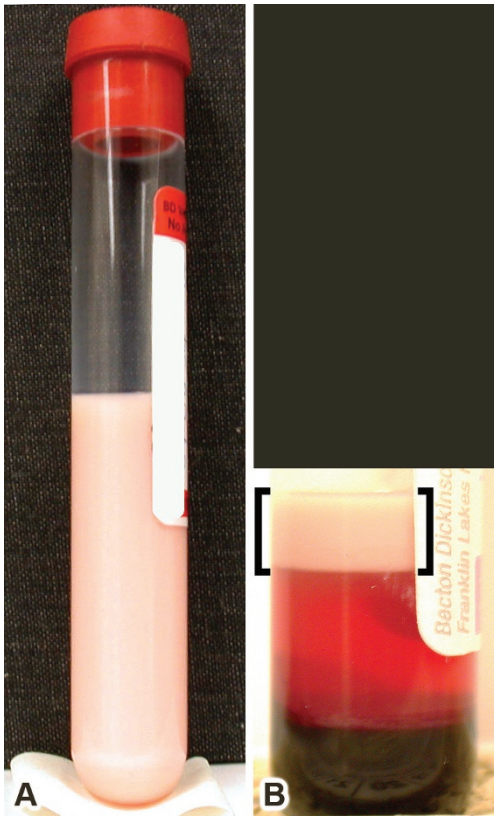


Figure 16.5. Lipemia. A. Lipemic serum: Marked lipemia and mild hemolysis in serum separated from blood cells by centrifugation. B. Chylomicron layer in blood: A thick chylomicron layer (*between brackets*) has formed over a blood sample after overnight refrigeration. The plasma between this "cream" layer and the cells is hemolyzed, as is common with stored lipemic samples.

C. *Secondary hyperlipoproteinemia* disorders are acquired disorders involving damaged cells or abnormal hormonal activity. They are relatively common in domestic mammals (Table 16.6).

III. Physiologic (postprandial) hyperlipidemia

A. This hyperlipidemia occurs in monogastric mammals and is caused by a hyperchylomicronemia that occurs after intestinal absorption of monoglycerides and FAs that have formed from digestion of ingested TG (Figure 16.3). Hypertriglyceridemia is present; hypercholesterolemia is mild or absent.

B. In healthy dogs and cats, the hyperchylomicronemia peaks 2–6 h after a meal, as assessed by [TG], and the chylomicrons are usually cleared by 16 h.[4,72] Delayed clearance of chylomicrons indicates defective lipoprotein metabolism, either a primary or secondary hyperlipidemia.

C. The presence of postprandial hyperlipidemia with and without addition of pancreatic extracts to food has been used to assess for exocrine pancreatic insufficiency (plasma

Table 16.6. Common serum cholesterol and TG findings in selected hyperlipoproteinemia disorders[1]

Disorder	[Chol.][a]	[TG][a]
Physiologic (postprandial)	WRI(–↑)	↑–↑↑↑
Primary and possibly primary		
Idiopathic hyperlipidemia of miniature schnauzers	WRI–↑	↑–↑↑↑
Idiopathic hyperlipidemia of Shetland sheepdogs	↑–↑↑↑	WRI–↑
Primary hyperchylomicronemia, type III LPL deficiency (cats)	WRI	↑↑↑
Idiopathic hypercholesterolemia (rough collies, briards)	↑	WRI
Other, single case reports (see the text)		
Secondary		
Hypothyroidism	↑–↑↑↑	↑
Hyperadrenocorticism	↑–↑↑	↑–↑↑
Diabetes mellitus	↑	↑–↑↑
Pancreatitis	WRI–↑	↑–↑↑
Protein-losing nephropathy and nephrotic syndrome	↑–↑↑↑	WRI–↑
Cholestasis	WRI–↑↑	WRI
Hepatic lipidosis in cats	WRI	↑–↑↑
Gram-negative sepsis	↓	↑
Transient hyperlipidemia in preweaned kittens	↑↑	↑↑
High dietary fat intake and obesity	WRI–↑	WRI–↑↑
Phenobarbital-associated	WRI	↑
Equine hyperlipemia (see the text)	WRI–↑	↑–↑↑↑
High catecholamine state in llamas and alpacas (see the text)	WRI–↑	↑–↑↑↑

[a] Cholesterol (Chol.) and TG are carried in lipoproteins: LDL and HDL are cholesterol rich; chylomicrons and VLDL are TG rich.

turbidity test; Table 15.11). However, variations in gastrointestinal function cause variations in the development and clearing of postprandial hyperlipidemia, thus complicating interpretation of the test. This test has been largely replaced by measurement of serum trypsin-like immunoreactivity (see Chapter 15).

IV. Primary and possible primary hyperlipidemia disorders in domestic mammals (Table 16.6)
   A. Idiopathic hyperlipidemia of miniature schnauzers[73]
      1. The pathogenesis of the hyperlipoproteinemia is not established.[74]
      2. There is hypertriglyceridemia with or more often without hypercholesterolemia. Prevalence and severity of hypertriglyceridemia increase with age.[73]
      3. Bismuth-NaEDTA lipoprotein density ultracentrifugation suggests that hyperlipidemic miniature schnauzers have increased VLDL with or without increased chylomicrons.[75] Differences in lipid profiles of hyperlipidemic miniature schnauzers were associated with different responses to a low-fat diet and therefore might reflect different subtypes of the disorder.[76]
      4. Hypertriglyceridemia may predispose affected miniature schnauzers to proteinuria (no hypoalbuminemia), gallbladder mucoceles, insulin resistance, and pancreatitis. In

one study, miniature schnauzers (n = 17) with a history of pancreatitis were 5 times as likely to have hypertriglyceridemia and 15 times as likely to have moderate to severe hypertriglyceridemia (> 500 mg/dL) as age-matched miniature schnauzers without pancreatitis.[77] [TG] was measured after resolution of clinical pancreatitis; therefore, pancreatitis-induced changes in lipid metabolism were considered unlikely.

B. Idiopathic hyperlipidemia occurring in Shetland sheepdogs[78,79]
   1. The pathogenesis of the hyperlipoproteinemia is not established.
   2. There is moderate to marked hypercholesterolemia, the prevalence of which increases with age. Concurrent hypertriglyceridemia is more common in older dogs.
   3. Lipoprotein electrophoretic patterns suggest an increase in one HDL subtype and LDL.

C. Primary hyperchylomicronemia in cats, type III LPL deficiency[80–82]
   1. Affected cats have a mutated LPL gene resulting in lack of plasma LPL activity (prior to and after heparin) but increased concentrations of the LPL protein (a type III LPL defect). This autosomal recessive hereditary defect causes defective lipolysis of plasma lipoproteins.
   2. Cats with this disorder have marked hypertriglyceridemia. Concentrations of cholesterol were not significantly different from those in unaffected cats in published reports. The persistent hyperlipidemia causes lipemia retinalis, formation of xanthomas in subcutaneous tissue and internal organs, and peripheral neuropathies because of nerve compression by xanthomas.

D. Idiopathic hypercholesterolemia in briards[83]
   1. The mean [cholesterol] in sera from 15 clinically healthy briards was 309 mg/dL compared to an age-matched and gender-matched group of nonbriards that had a mean [cholesterol] of 159 mg/dL, but [cholesterol] was not necessarily increased above common canine URLs.
   2. The briards had increased α_2-lipoprotein concentrations that suggested increased [HDL]. [TG] was not abnormal.
   3. The hyperlipoproteinemia appears to be familial, but until the defect is known, classification as a primary disorder is premature. The healthy briards were evaluated to explore the cause of lipid droplet accumulation associated with retinal pigment epithelial dystrophy in the breed, and the results of the study suggested a possible relationship. The genetic defect in the retinal lesion has been established, but the relationship to hypercholesterolemia (if any) is still not understood.

E. Idiopathic hypercholesterolemia in rough collies[84]
   1. Increased or high-normal [cholesterol] was found in five related rough collies with corneal lipidosis.
   2. Because no secondary cause was identified, the disorder may be primary. The severity of the ocular lesions was not related to the magnitude of hypercholesterolemia.

F. Golden retriever muscular dystrophy[85]
   1. Affected dogs, and less so, carrier dogs, reportedly have increased cholesterol and TG concentrations compared to healthy dogs, but most reported values were probably WRI (RIs not provided).
   2. The investigators suggested that the lipid changes might reflect a primary dyslipidemia, but a role for dystrophin in lipoprotein metabolism is not described.

G. Other, single case reports
   1. A 5-yr-old spayed female West Highland white terrier with neurologic signs had persistent marked hypercholesterolemia and moderate hypertriglyceridemia that was

suspected to reflect a primary hyperlipidemia because no other cause was found.[86] Magnetic resonance imaging of the brain was consistent with multiple ischemic strokes which have been associated with severe hypercholesterolemia.

2. A 2.5-yr-old castrated male miniature American shepherd had persistent marked hypercholesterolemia, marked hypertriglyceridemia, and marked atherosclerosis that appeared to cause ischemic damage resulting in cutaneous lesions and behavioral change.[87] A primary hyperlipidemic disorder was suspected given lack of other antemortem or postmortem evidence of another cause.
3. Congenital LPL deficiency in a mixed-breed pup:[88] A 1-mo-old mixed-breed dog had marked hypertriglyceridemia (830 mg/dL), hypercholesterolemia (312 mg/dL), and normoglycemia in a serum sample that looked like cream-of-tomato soup. The pup was the smallest dog of the litter and died a few days after initial presentation. LPL deficiency was diagnosed by the failure of intravenous heparin (3000 IU) to produce fatty-acid changes consistent with lipolytic activity.
4. Hyperlipidemia in a Brittany dog:[89] A 9-yr-old male Brittany dog was suspected to have "hyperlipemia due to a progressive inactivation of LPL with aging." Hypertriglyceridemia was found at 5, 7, and 9 yr of age, and hypercholesterolemia was not present. Serum [TG] did not decrease after heparin administration. The hyperlipidemia was considered a primary disorder because a secondary cause was not found and the dog's son (3 yr younger) also had fasting hypertriglyceridemia. However, response to heparin in the son was similar to a control dog's response.
5. One of the first reports of hyperlipoproteinemia in beagles[90] is sometimes cited as a form of primary hyperlipoproteinemia, but the investigators did not exclude hypothyroidism as a cause.
6. Primary hypercholesterolemia was suspected in a 5-yr-old cat with unexplained hypercholesterolemia and a cutaneous xanthoma.[91]

V. Secondary hyperlipidemia disorders (Table 16.6)
   A. Dogs and cats
      1. Canine hypothyroidism
         a. About 75 % of dogs with hypothyroidism have mild to marked fasting hypercholesterolemia.[11,92] Although hypertriglyceridemia has also been reported in many affected dogs, it has not been shown to be as helpful as hypercholesterolemia in distinguishing hypothyroid dogs from euthyroid dogs.
         b. Because some forms of hypothyroidism are familial, the associated hyperlipoproteinemia is familial.[93,94] However, the defect in lipid metabolism is a secondary hyperlipoproteinemia because it is caused by decreased thyroid hormone activity.
         c. In human hypothyroidism, the hyperlipidemia appears to be related primarily to decreased expression of hepatic LDL receptors, which capture LDL, and decreased activities of LPL and hepatic lipase.[95,96]
            (1) Low [thyroxine] is associated with low LPL activity. Thus, reduced intravascular lipolysis of chylomicrons and VLDL may cause hypertriglyceridemia.
            (2) Decreased hepatic lipolysis and uptake of LDL causes hypercholesterolemia.[97] Data in people have shown a correlation between serum [triiodothyronine] and hepatic lipase activity.
      2. Hyperadrenocorticism (spontaneous and iatrogenic)
         a. Dogs with spontaneous hyperadrenocorticism often have fasting hypercholesterolemia, and they may have hypertriglyceridemia as well, though

published data on prevalence are scarce. In one study of dogs with iatrogenic hyperadrenocorticism, 12 of 18 had hypertriglyceridemia and 14 of 28 had hypercholesterolemia.[98]

b. Hypercholesterolemia occurs in some cats with hyperadrenocorticism. In a study involving 30 cats with hyperadrenocorticism, 9 of 24 had hypercholesterolemia and 5 of 7 had hypertriglyceridemia.[99]

c. Glucocorticoid administration to cats: Cholesterol increased above baseline in cats treated with prednisolone or dexamethasone, enough to become hypercholesterolemic in some cats, particularly those treated with dexamethasone.[100] The [TG] increased from baseline, but not above the URL, in all treated cats. In a study of cats with allergic dermatitis, hypertriglyceridemia developed during administration of anti-inflammatory doses (1 or 2 mg/kg/d) of prednisolone for 7 d.[101]

d. Because increased glucocorticoid activity may lead to diabetes mellitus or steroid hepatopathy, it may be difficult to determine which pathologic state is causing the hyperlipidemia.

e. Effects of glucocorticoid hormones (endogenous and exogenous) on lipid metabolism are multifactorial and poorly understood, but direct and indirect effects on LPL and hormone-sensitive lipase activities, increased FA production, and increased VLDL synthesis may contribute to hypertriglyceridemia and hypercholesterolemia in patients with hyperadrenocorticism.[102–104]

3. Diabetes mellitus

a. Many diabetic dogs and cats have hypertriglyceridemia, typically with hypercholesterolemia.[4,105] The pathogenesis and severity of the hyperlipoproteinemia may vary with the type of diabetes mellitus (see Chapter 14).

b. In diabetic dogs with or without concurrent disorders, hypercholesterolemia occurred with increases in concentrations of cholesterol within VLDL, HDL, and especially LDL fractions.[105]

c. Hypercholesterolemia in cats with acromegaly or hyperadrenocorticism has been attributed to the diabetic state arising from these conditions.[106]

d. The hyperlipoproteinemia may be caused by multiple mechanisms, including decreased intravascular lipolysis and increased production of TG-rich lipoproteins:[4,107]

(1) Defective intravascular processing of chylomicrons and VLDL may occur because of low [insulin], high [NEFA], and thus altered LPL activity.

(2) Increased VLDL synthesis by hepatocytes may occur due to mobilization of FAs that is caused by increased hormone-sensitive lipase activity in adipocytes. The influx of FAs into hepatocytes and their oxidation causes excess formation of acetyl-CoA that promotes TG, cholesterol, and ketone production.

(3) Hepatic cholesterol synthesis was increased in an experimental model of canine diabetes mellitus with increases in LDL and decreases in HDL. The appearance of a modified HDL (HDLc) suggests that changes in reverse cholesterol transport may contribute to dyslipidemia.[108,109]

(4) There is increased synthesis of HDL cholesterol by intestinal mucosal cells in experimental canine diabetes mellitus.[110]

4. Pancreatitis

a. Dogs with acute pancreatitis frequently have hypertriglyceridemia with or without hypercholesterolemia. In one small study, 12 of 28 (43 %) dogs with pancreatitis had hypertriglyceridemia, hypercholesterolemia, or both, but only 29 % (5 of 17)

had these changes after excluding dogs with concurrent disease that could influence lipid metabolism.[111] Most of the increases in dogs with only pancreatitis were mild, so marked increases in dogs with pancreatitis suggest concurrent disease.

b. Hypertriglyceridemia may be an initiating factor or a consequence of pancreatitis, the former being supported by the fact that miniature schnauzers with a history of pancreatitis were more likely to have hypertriglyceridemia than age-matched controls without pancreatitis.[112]

c. The pathogenesis of hyperlipidemia and pancreatitis-associated changes in lipoproteins is not firmly established and may be multifactorial.[111,113]

(1) As noted, it may be caused by an underlying dyslipidemia or concurrent disorders such as diabetes mellitus and hyperadrenocorticism.[114,115] Hypoinsulinemia or insulin resistance cause increased lipolysis in adipose tissue followed by increased hepatic synthesis of VLDL, which are transformed to LDL in the bloodstream.

(2) TNF and other cytokines have been shown to alter lipoprotein metabolism and result in increased [VLDL].[116,117] Thus, the lipemia of pancreatitis could be associated with the release of cytokines during inflammation.

(3) Because pancreatitis may cause obstructive cholestasis, part of the hyperlipidemia may relate to defects in lipid metabolism caused by cholestasis.

5. Protein-losing nephropathy and nephrotic syndrome

a. Hypercholesterolemia can occur in progressive protein-losing nephropathy and is one of the defining criteria of nephrotic syndrome. Hypertriglyceridemia is an inconsistent but common finding in people with nephrotic syndrome;[118] little information is reported for dogs and cats, but [TG] in three dogs with nephrotic syndrome was not greater than that in dogs with nonnephrotic glomerular disease.[119]

b. Studies in rats and mice reveal that hyperlipidemia results from decreased lipoprotein catabolism, decreased hepatic uptake of lipids, and increased synthesis.[118] These alterations are largely a consequence of hypoalbuminemia and proteinuria.

(1) Decreased catabolism of TG-rich lipoproteins likely results mostly from decreased endothelial LPL activity.[120,121]

(2) Decreased hepatic uptake and processing of lipoproteins by the liver occurs because of reduced expression of lipoprotein receptors and reduced activity of hepatic lipase.[118]

(3) Increased FA and cholesterol synthesis is related to up-regulation of acyl-CoA: cholesterol acyltransferase activity caused by proteinuria (not hypoalbuminemia);[122] this leads to esterification of cholesterol in the liver. The resulting decrease in intrahepatic free cholesterol leads to upregulation of enzymes involved in both TG and cholesterol synthesis.[118]

(4) Decreased proportions of HDL have been documented in dogs with nephrotic syndrome.[123] In people, a similar finding is reported to be a consequence of urine loss of LCAT, an enzyme involved in maturation of HDL.

c. Dogs and cats with protein-losing nephropathy may have hypoalbuminemia and hypercholesterolemia but insufficient sodium and H_2O retention to cause the ascites and edema required to fulfill traditional criteria for a diagnosis of nephrotic syndrome.

d. Dogs with chronic renal disease but not nephrotic syndrome may have a similar dyslipidemia with increased proportions of VLDL and LDL and a decrease in the proportion of HDL, with or without hypercholesterolemia.[123] TG concentrations were not measured.

6. Cholestasis
   a. Dogs and cats with intrahepatic and extrahepatic cholestatic disorders frequently but inconsistently have hypercholesterolemia, generally without concurrent hypertriglyceridemia.[124–126] The nature and severity of the underlying disorder may influence whether hypercholesterolemia develops.
      (1) Hypercholesterolemia was present in 56 % of 43 tested dogs with cholangitis or cholangiohepatitis (mostly neutrophilic), and 64 % of tested dogs were hyperbilirubinemic, supporting cholestasis.[127] [TG] was not reported.
      (2) In a study of 45 dogs with chronic lymphoplasmacytic hepatitis and no concurrent disease known to cause hyperlipidemia, a majority of dogs had hypertriglyceridemia (33 %), hypercholesterolemia (11 %), or both (18 %), but hypertriglyceridemia was also present in 11% of dogs considered healthy.[128] No bilirubin data were provided to help assess a relationship with cholestasis, but hypercholesterolemia was associated with increased GGT activity and with moderately to severely increased ALP activity, abnormalities suggesting cholestasis.
      (3) Hypercholesterolemia and hypertriglyceridemia are associated with biliary mucoceles in dogs; however, it is not clear whether the hyperlipidemia is a cause or a consequence of concurrent cholestasis. Shetland sheepdogs and miniature schnauzers, two breeds in which primary hyperlipidemias have been recognized, are prone to mucoceles.[129,130]
   b. The hypercholesterolemia of cholestasis is associated with increased cholesterol content of hepatocytes, and it appears to result from multiple processes.
      (1) Cholestasis reduces biliary excretion of cholesterol from hepatocytes.
      (2) There is experimental evidence in rats and mice for increased cholesterol synthesis during cholestasis.[131,132]
      (3) In experimental cholestasis in dogs, increased [LDL] suggests a defective uptake of LDL by hepatocytes.[133]
   c. Cholestasis has been associated with the appearance of lipoproteins X1, X2, and X3 that have cathodal electrophoretic migrations.
      (1) The formation of lipoprotein X is poorly understood, but it is thought to involve increased hepatic phospholipid and cholesterol synthesis.[134] These lipoproteins have a similar density to LDL, but they have a lipid content predominated by phospholipids and nonesterified cholesterol, and they have a small protein content dominated by albumin within the core and apolipoprotein C on the surface. Unlike VLDL and LDL, they have little or no apolipoprotein B and therefore do not undergo LDL receptor-mediated hepatocyte uptake.[135] They are cleared mostly by macrophages.
      (2) Lipoprotein X cholesterol is commonly mistaken for LDL cholesterol in human samples, which is significant because lipoprotein X is nonatherogenic, as opposed to LDL, which is atherogenic and responsive to statins.
      (3) Some investigators have reported the presence of lipoprotein X in cholestatic dogs, but in other studies of experimental cholestasis in dogs, none of the increased lipoprotein fractions had a composition characteristic of human lipoprotein X.[133,136–138]
7. Hepatic lipidosis in cats
   a. Moderate to marked hypertriglyceridemia has been demonstrated in some clinical cases and experimental models of feline hepatic lipidosis, but [TG] is often WRI despite an increase in the ratio of LDL to HDL.[53,139–141] [Cholesterol] is typically WRI or only mildly increased.

b. The pathogenesis of the dyslipidemia is not known. Increases in total amounts of VLDL (some studies) and LDL, and TG-enrichment of LDL suggest that there is increased hepatic lipoprotein synthesis and secretion concurrent with decreased lipolysis of these lipoproteins, and hepatic lipid accumulation suggests that synthesis exceeds secretion.[139–141] Lipoprotein synthesis may be promoted by increased FAs released from adipose tissue because of increased activity of hormone-sensitive lipase, which could be secondary to hypoinsulinemia or increased production of cortisol, catecholamines, or glucagon.[69]

8. Gram-negative sepsis: Although poorly documented in domestic mammals, Gram-negative sepsis may cause hypertriglyceridemia in addition to hypocholesterolemia (see Cholesterol Concentration, Section IV.B.2.b).[142,143] Mechanisms are thought to include increased hepatic production of TG, decreased intravascular hemolysis because of LPL inhibition, and decreased hepatic uptake because of defenestration (decreased porosity) of hepatic sinusoidal endothelial cells.
9. Transient hyperlipidemia in preweaned kittens:[144,145] Affected 3- to 8-wk-old kittens have had marked lipemia with marked hyperchylomicronemia, hypercholesterolemia, and hypertriglyceridemia. Some have died or undergone euthanasia related to severe hepatic lipidosis and multi-organ failure. Others have recovered rapidly with forced high-protein nutrition, supporting pathogenic roles for negative energy balance and protein malnutrition in this disorder.
10. High dietary fat intake and obesity
    a. Although the quantity and type of fat in diets may alter lipoprotein content, fasting [cholesterol] and [TG] are not anticipated to exceed their respective URLs in animals fed commercial diets.[4,146]
    b. Excessive dietary lipid can cause obesity and prolonged postprandial hyperlipidemia because of insulin resistance or other factors, and extremely elevated fat intake with concurrent inadequate protein intake can lead to hepatic lipidosis, defective lipoprotein metabolism, and altered lipoproteins.
    c. Obese dogs and cats may, on average, have greater [TG] and [cholesterol] than nonobese dogs and cats, and both values can be above URLs in individuals, but the values are often WRI.[147,148]
11. Phenobarbital-associated: One third of epileptic dogs treated for at least 3 mo with phenobarbital, with or without potassium bromide, had fasting hypertriglyceridemia associated with hyperchylomicronemia.[149] The pathogenesis of the apparent delay in chylomicron clearance was not determined, but drug-induced polyphagia and weight gain may have contributed.

B. Equids: hyperlipidemias of horses, ponies, and donkeys
1. Terms related to dyslipidemias of horses have varied, so definitions that align with common, current use are provided and used here.[150] *Hypertriglyceridemia* is an increase in serum or plasma [TG]. *Lipemia* refers to the lactescent appearance of serum or plasma, which may not occur in horses with marked hypertriglyceridemia; mild hypertriglyceridemia (< 300 mg/dL) has little clinical consequence and occurs in fasting horses as a result of negative energy balance.[71] *Severe hypertriglyceridemia* has been defined as [TG] > 500 mg/dL without lipemia. *Hyperlipidemia* is any increase in serum or plasma lipids (usually in the context of hypertriglyceridemia). In contrast, *equine hyperlipemia* or *hyperlipidemic syndrome* refer to the pathologic state of hyperlipidemia associated with lipemia, hepatic lipidosis, and clinical disease.[151,152]
2. Equine hyperlipemia is seen most commonly in ponies and donkeys and has been considered a primary disorder in these equids because an underlying disorder is not

always detected; however, a specific genetic abnormality has not been reported. Marked hyperlipidemia in full-size horses is uncommon and usually occurs secondary to an underlying disorder. Hyperlipemia is reportedly more common in miniature horses, although unlike in ponies, a concurrent disease is frequently identified.[153] Equine hyperlipidemias are discussed here as secondary disorders because predisposing factors and concurrent diseases are recognized, especially in horses.[154,155]

3. Ponies, donkeys, and miniature horses with marked hypertriglyceridemia typically have lipemic serum; however, full-size horses with marked hypertriglyceridemia secondary to severe inflammatory disorders may not. The reason for this discrepancy is not known. Lack of serum or plasma lactescence impairs recognition of hypertriglyceridemia in large-breed equids.[71]
4. Mild to moderate hypercholesterolemia may accompany hypertriglyceridemia in equids with hyperlipemia.[156]
5. Although predisposing causes and underlying conditions vary, marked hyperlipidemia results from increased synthesis of TG-rich lipoproteins by the liver:[156]
   a. Negative energy balance stimulates hormone-sensitive lipase in adipocytes to increase release of FAs. Glucocorticoids released during physiologic stress also stimulate hormone-sensitive lipase and cause insulin resistance.
   b. FAs are transported to the liver at a rate exceeding the liver's ability to oxidize them for energy. Hepatocytes subsequently increase the synthesis of TG-rich VLDL that enters blood to cause hypertriglyceridemia. If the condition is severe enough, TG accumulates in hepatocytes causing hepatic lipidosis and organ damage. Lipid also may accumulate in renal tubules.
   c. Decreased insulin concentration (associated with fasting or late gestation) or decreased insulin activity and insulin resistance (associated with breed, obesity, stress, pregnancy, lactation, etc.) may reduce insulin's negative effect on hormone-sensitive lipase and thus promote FA release.[157]
   d. Decreased intravascular lipolysis does not appear to play a role as once thought; activity of LPL and hepatic lipase are increased in ponies during hypertriglyceridemia due to both physiologic and pathologic causes.[156]
6. Potential predisposing factors in ponies and donkeys with hyperlipidemia, including hyperlipemia, are female sex, breed, older age, obesity, pregnancy, lactation, and calorie restriction.[154,156,158] Furthermore, there is evidence of inherent breed-related differences in insulin responses to glucose that supports a genetic predisposition to insulin resistance in ponies and certain other equids (e.g., Andalusian horses).[159,160]
7. Underlying and associated disorders[71]
   a. Reported disorders leading to negative energy balance in horses and foals with marked hyperlipidemia include gastrointestinal disease, hepatic disease, and infections. Cytokines such as TNF and interleukin-6 may play roles in the marked hyperlipidemia because many of these horses have signs of a systemic inflammatory response.[117,161,162] Some references cite renal disease as an important cause of hyperlipemia;[163] however, studies reporting azotemia in hyperlipidemic horses do not consistently distinguish between prerenal and renal azotemia. In addition, renal injury may be a consequence of renal lipidosis.
   b. Endocrine causes of equine hyperlipemia
      (1) Pituitary pars intermedia dysfunction, type 2 diabetes mellitus, or both, in horses and ponies:[164] Reported individuals have had marked hypertriglyceridemia accompanied by weight loss despite a good appetite. The pathogenesis is unknown, but roles for insulin resistance and an increase in catabolic- and lipolysis-promoting hormones are expected.

(2) Equine metabolic syndrome is defined by obesity, insulin resistance, and predisposition to laminitis. Horses with equine metabolic syndrome often have dyslipidemia characterized by hypertriglyceridemia.[165]

c. Equine pancreatitis: Hyperlipemia and hypertriglyceridemia were present in two foals with histologically confirmed pancreatitis and no evidence of sepsis.[166]

C. Cattle: Unlike other species, cattle do not develop hypertriglyceridemia in states of negative energy balance, but instead have increased plasma [NEFA] and ketonemia. This is likely a result of a comparatively reduced capacity of hepatocytes to assemble or secrete VLDL.[10]

D. Llamas and alpacas[65,167,168]

1. Hypertriglyceridemia develops secondary to many metabolic, endocrine, and infectious diseases, e.g., diabetes mellitus, endoparasitism and diarrhea, hepatic lipidosis, neonatal sepsis, enteritis, and infection with "*Candidatus* Mycoplasma haemolamae." Marked hypertriglyceridemia leading to grossly lipemic serum is often accompanied by increased [NEFA] and [β-hydroxybutyrate]. Hypercholesterolemia is less common and, if present, of lesser magnitude.
2. Unlike in horses, hypertriglyceridemia is not associated with sex, age, or reproductive status.[167]
3. Llamas and alpacas with hypertriglyceridemia are often normoglycemic or even hyperglycemic, suggesting that factors other than negative energy balance and carbohydrate insufficiency play a role. By contrast, cattle are typically hypoglycemic during states of marked lipid mobilization resulting from negative energy balance.
4. Hypertriglyceridemia can be initiated by catecholamines, with increased epinephrine stimulating glycogenolysis, lipolysis, hepatic lipoprotein synthesis, and ketogenesis beyond what is typical during fasting.[169]

OTHER ASSESSMENTS OF LIPIDS

Limited availability of standardized and validated methods for differentiating lipoprotein classes hinders recognition of some dyslipidemias in veterinary medicine.[75,170] Because of differences in lipoproteins among species, methods designed and optimized for human specimens require careful validation in veterinary species. The following is a summary of common methods for analyzing classes of lipoprotein. The challenges of analyzing lipoproteins, a diverse class of molecules, are summarized in reference texts.[1]

I. Lipoprotein electrophoresis: Lipoprotein electrophoresis is used as an adjunct qualitative assessment of lipoprotein abnormalities in clinical veterinary samples. However, methods vary, and test availability and interpretive expertise are limited.

A. The following is a brief summary of the standard procedure:

1. EDTA plasma (or serum) is applied to a gel (usually agarose). An electric current causes lipoproteins to migrate through the gel based on their charge, size, shape, and interaction with the gel.
2. After separation, lipid stains are applied to visualize the lipoprotein bands, which are named according to serum α- and β-protein bands of corresponding mobility.
   a. Chylomicrons, if present, remain at the application origin.
   b. LDL and VLDL molecules migrate intermediately (β or pre-β bands).
   c. HDL molecules migrate farthest toward the anode (α bands).
3. The gel should be examined qualitatively for dyslipoproteinemic patterns. In dogs, increased α_2-migrating lipoproteins are associated with hypercholesterolemia, and

increased pre-β bands, β bands, or application site bands are associated with hypertriglyceridemia.[171]

4. Although gel banding patterns can provide useful information about lipoprotein subfractions, bands are not consistently associated with certain classes of lipoproteins as defined by their densities. For example, canine lipoproteins with densities of 1.063–1.087 separate into α- and β-migrating bands identified as HDL and LDL, respectively.[2] Therefore, lipoprotein electrophoresis is most useful when combined with density fractionation studies or when used to monitor response to therapy.
5. A study comparing one automated and one manual method for canine serum lipoprotein electrophoresis revealed substantial differences in banding patterns and lack of agreement regarding relative amounts of α, pre-β, and β fractions. The pre-β (VLDL) fraction was poorly detected by the automated method.[172]

B. A nondenaturing linear polyacrylamide gel electrophoresis system (Lipoprint) designed to fractionate human serum or plasma lipoproteins has been explored for use in dogs, horses, and cattle.[173,174] Heparin should not be used as the anticoagulant because it interferes with LDL fractionation.[175]

1. Samples are added to liquid loading gel containing a dye that binds the cholesterol component of lipoproteins. Quantitation of subfractions requires sample [cholesterol] > 100 mg/dL so that all the dye is bound to cholesterol.
2. Loading gel is layered over stacking and separating gels in HDL- or LDL-fractionation tubes and then photopolymerized. When current is applied, lipoproteins separate based on size and charge, with HDL migrating furthest, VLDL least, and chylomicrons remaining in the stacking or loading gel.
3. Gels are scanned densitometrically for cholesterol staining to yield tracings subdivided into lipoprotein fractions and subfractions. Software calculates the area under the curve for each, allowing calculation of the percentage each fraction is of the total. For domestic mammals, the lipoproteins in each fraction have not been characterized by other methods.

II. Ultracentrifugation: Ultracentrifugation of hydrated lipoprotein using established densities followed by component analysis is another method for measuring and characterizing lipoprotein classes. However, density limits defining human lipoprotein fractions[1] are not broadly applicable, and because of overlapping densities of functional lipoprotein classes in some veterinary species (Figure 16.1), their complete separation cannot be achieved by this method. However, continuous lipoprotein density profiling with ultracentrifugation using bismuth-NaEDTA to create a density gradient, and with a fluorescent sphingosine probe to label lipoproteins, has been used to assess lipoprotein density distributions in healthy and sick dogs, cats, and horses.[53,111,139,176]

III. Chromatography: Chromatography and related methods that combine separation and fluorescent-based lipid measurements have the advantage of smaller sample size and rapid run times that may change the analysis of lipoproteins in veterinary medicine in the future.[177] The method has been used to evaluate lipoproteins in dogs and cats.[53]

IV. Assays for HDL-cholesterol and LDL-cholesterol concentrations

A. Although frequently described as measurements of HDL and LDL, these assays measure serum concentrations of cholesterol carried in HDL and in LDL, so-called good and bad cholesterol, respectively. Because HDL-cholesterol and LDL-cholesterol decision limits have important implications for risk-assessment and medical intervention in human

medicine, rapid analytical methods were developed and are used routinely for human patients.

B. Human HDL-cholesterol and LDL-cholesterol concentrations can each be measured by indirect and direct methods. Indirect methods require manipulation or manual separation of lipoproteins prior to measuring cholesterol concentration in the subfractions, and some of these are the reference methods. In contrast, direct methods, which are also called homogeneous methods, are more clinically applicable "mix and read" assays utilizing various chemical and immunologic reagents to isolate the desired lipoprotein and measure its [cholesterol] in one reagent/sample mixture. Direct assays have been widely automated.

C. Automated-direct assays have variable performance compared to reference methods and perform better in health than in disease.[1] For example, the most common method used to determine human [LDL cholesterol] is by a calculation that incorporates a factor designed to correct for the contribution of cholesterol in VLDL. By this approach, results of direct assays for [total cholesterol], [TG], and [HDL cholesterol] are entered into the Friedewald equation: [LDL cholesterol] = [total cholesterol] – [HDL cholesterol] – ([TG]/5), where all values are in mg/dL and the "[TG]/5" factor corrects for the small amounts of cholesterol normally contributed by VLDL.[178] This correction factor is inappropriate in patients with hypertriglyceridemia, chylomicronemia, and increases in certain abnormal VLDL, and it is unlikely applicable to all species.

D. To the authors' knowledge, no direct assays for HDL-cholesterol or LDL-cholesterol concentrations have been validated for use in domestic mammals, and one assay for human HDL-cholesterol concentration was shown to be inadequate for bovine samples.[179]

V. Standing plasma test for chylomicrons (also called a refrigeration test)

A. Because of their high TG content and thus low density, chylomicrons float to the top of a serum or plasma sample if the sample is undisturbed (Figure 16.5). A 16-h–standing test has been used to detect chylomicrons.

B. In the procedure, 2 mL of plasma is placed in a small glass tube and then examined after 16 h of refrigeration at 4 °C.

1. The presence of a creamy layer on the surface of the plasma sample indicates the presence of chylomicrons. Chylomicrons indicate postprandial hyperlipidemia (which could be pathologic if it represents delayed clearing).
2. Turbidity below or without a surface creamy layer suggests that other causes of hyperlipidemia may need to be considered.

C. However, the 16-h–standing test has relatively poor capability of detecting chylomicrons when compared to lipoprotein electrophoresis and ultracentrifugation. In one study, only about 20 % of the chylomicron-positive samples (as detected by electrophoresis) had a positive 16-h–standing test.[180] Similar results were found when the standing test was compared to ultracentrifugation.[181]

D. The presence of a creamy layer in the standing test is specific for chylomicrons, but many samples that contain chylomicrons do not have a detectable lipid layer.

References

A complete set of references is available online. Please use the below link to access the References files.

https://www.wiley.com/go/fundamentals/vet

Chapter 17

THYROID FUNCTION

Michael A. Scott and Steven L. Stockham

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Table 17.1. Abbreviations and symbols in this chapter	
$[fT_4]_{ed}$	Free thyroxine concentration by equilibrium dialysis
[x]	Concentration of x (x = analyte)
ALP	Alkaline phosphatase
fT_3	Free triiodothyronine
fT_4	Free thyroxine (tetraiodothyronine)
M_r	Relative molecular mass
NTI	Nonthyroidal illness
RI	Reference interval
RIA	Radioimmunoassay
SI	Système international d'unités (International System of Units)
T_3	Triiodothyronine
T_3AA	Triiodothyronine autoantibodies
T_4	Thyroxine
T_4AA	Thyroxine autoantibodies
TgAA	Thyroglobulin autoantibodies
TpAA	Thyroid peroxidase autoantibodies
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone (thyrotropin)
tT_3	Total triiodothyronine
tT_4	Total thyroxine
WRI	Within reference interval

PHYSIOLOGIC PROCESSES

I. Hormonal control of thyroid glands (Figure 17.1)
   A. TRH is a tripeptide from the hypothalamus that stimulates the production and release of TSH from thyrotrope cells in the anterior pituitary gland and also acts as a neurotransmitter in extrahypothalamic regions of the brain.[1] Increased $[T_3]$ and decreased leptin concentration inhibit the release of TRH, whereas decreased $[T_3]$ and increased leptin concentration stimulate the release of TRH.[2] Tanycytes, specialized ependymal cells lining the third ventricle, extend long processes into the hypothalamus where they interact with axons containing TRH and participate in control of TRH release.
   B. TSH (thyrotropin) binds to thyroid TSH receptors on thyroid follicular cells and stimulates the production and release of T_4 (mostly) and T_3 from the thyroid glands. All circulating T_4 is produced by the thyroid glands, but a minority (10–40 %) of circulating T_3 is produced by the thyroid glands.[3] The rest of T_3 is generated from T_4 in cells throughout the body; in this context, T_4 is a prohormone. In the pituitary gland, T_3 produced by the deiodination of T_4 inhibits secretion of TSH.
      1. Synthesis of T_4 and T_3 is also influenced by intrathyroidal factors, including availability of iodide and sensitivity to TSH.
      2. Thyroglobulin is produced by luminal thyroid cells and is stored in follicular lumina. Thyroglobulin acts as an acceptor protein for iodine and is the source of T_4 and T_3.

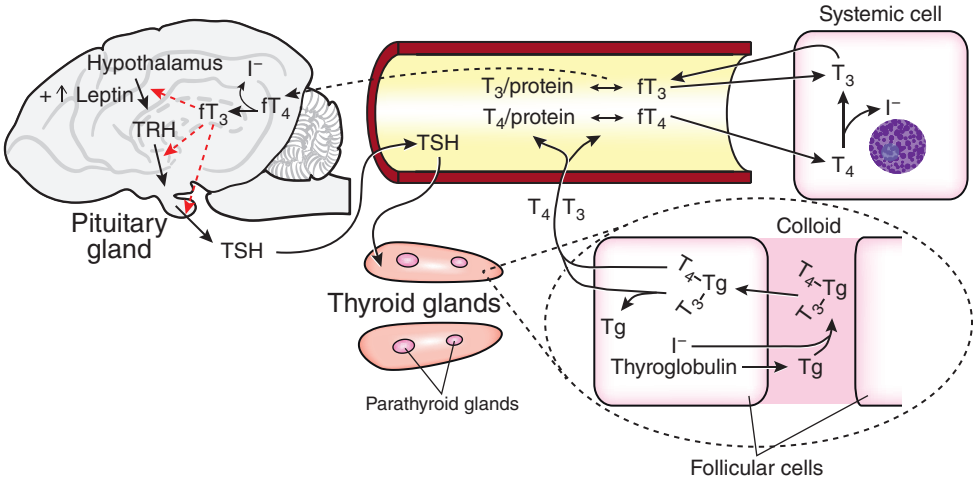


Figure 17.1. Hormonal control of thyroid glands.

- TRH released from the hypothalamus stimulates the release of TSH from the pituitary gland.
- TSH stimulates the production and release of T_4 and T_3 in pathways involving thyroglobulin in the thyroid glands.
  - Thyroglobulin is synthesized in follicular cells and is released into the luminal colloid by exocytosis.
  - Iodide (I^-) is transported from blood into the follicular cells via a Na^+/I^- symporter, and then into the colloid where it is oxidized to reactive iodine species by hydrogen peroxide and thyroid peroxidase on apical membranes of follicular cells. The reactive iodine species then rapidly bind to tyrosyl residues of thyroglobulin, a process called *organification* of thyroglobulin. Iodinated tyrosyl groups join together to form about 30 T_4 and few T_3 units within each thyroglobulin molecule. Thyroglobulin is stored within thyroid follicles, providing enough hormone for weeks to months.
  - The iodinated thyroglobulin enters follicular cells by pinocytosis of colloid, and the pinocytic vesicles fuse with lysosomes containing proteases that free T_4 and T_3 molecules from some of the thyroglobulin molecules.
- T_4 and T_3 are released from follicular cells and diffuse to blood via a rich network of fenestrated capillaries. Iodine within thyroglobulin tyrosyl groups that do not form T_4 and T_3 is recycled by a deiodinase enzyme. In blood, most T_4 and T_3 molecules are protein bound, but fT_4 and fT_3 can enter systemic cells where some of the T_4 is converted to T_3. T_3 has more activity than T_4. In the negative-feedback system (*red dashed lines*), fT_3 produced locally from fT_4 inhibits the secretion of TSH and TRH. Leptin stimulates the release of TRH; conditions that lower leptin concentrations inhibit TRH secretion.

3. Physiologic variations in [tT_4] have been reported. Age-related differences are addressed along with causes of hyper- and hypothyroxinemia (see Thyroxine (T_4) Concentration, Sections I.A.7 and I.B.5), but [tT_4] is generally high in neonates and declines with age.
   a. In horses, the highest concentrations are in the late afternoon, and the lowest concentrations are in the early morning. Pregnant and nonpregnant thoroughbred mares had a mild seasonal variation in [tT_4] with greater values related to lactation and without a statistically significant difference related to pregnancy.[4]
   b. In dogs, some authors report that there is not a predictable diurnal variation, and there can be confusing fluctuations in random baseline [tT_4]. However, in a study of a small group of dogs, [tT_4] was significantly lower at 8 a.m. (mean, 1.75 µg/dL) than at 11 a.m., 2 p.m., 5 p.m., and 10 p.m. The highest mean [tT_4] was at 2 p.m. (3.54 µg/dL).[5] A similar pattern (but with less change) was

found in 8 a.m., 2 p.m., and 8 p.m. samples.[6] Thus, the time of sampling may influence the interpretation of [tT_4]. Similar patterns were seen in [fT_4]$_{ed}$. In bitches, [tT_4] and [fT_4] tended to be somewhat higher and [TSH] lower in early to mid-pregnancy.[7] Physiologic breed differences are substantial, thus making use of population-based RIs problematic and consideration of breed and clinical signs important when interpreting results.[8–13]

c. Limited studies of the biological variation of feline thyroid hormone concentrations have yielded varied results, but they suggest that clinically significant individual changes in [tT_4], [fT_4], [fT_4]$_{ed}$, and [TSH] might be detected better by comparison to individual baseline values than with typical population-based RIs.[14,15] Different cat populations and assay methods likely contributed to differences in study results.

II. In blood, most tT_4 and tT_3 molecules are protein bound.

A. Nearly all T_4 released from the thyroid glands becomes bound to plasma proteins, providing a ready reserve when needed.[16] The small unbound portion is called fT_4. Thyroxine-binding globulin, transthyretin, and albumin are the three major carriers, with little T_4 bound to apolipoproteins, but there are species differences. In horses and cattle, T_4 appears to bind to thyroxine-binding globulin, transthyretin, and albumin, but thyroxine affinity chromatography failed to identify thyroxine-binding globulin in cats.[16,17] Although T_4 binds to transthyretin and albumin in dogs, the role of thyroxine-binding protein is not clear. One group reported that the canine gene for the thyroxine-binding protein is not expressed because of a promoter defect that prevents transcription.[16] Another group isolated a canine serum thyroxine-binding globulin by thyroxine affinity chromatography and considered it thyroxine-binding protein, although it had several differences from the human protein: (i) greater M_r (70,000 instead of 54,000), (ii) lack of immunodetection by an antibody to human thyroxine-binding protein, and (iii) circulation at much lower concentration.[18] A third group reported detection of a protein ($M_r \cong 51{,}000$) in canine serum that they "postulated" to be thyroxine-binding protein.[19] Whether dogs have a form of thyroxine-binding globulin or not, plasma [tT_4] in dogs and cats is roughly one-tenth that in people, while magnitudes of [fT_4] are similar, making the [tT_4]:[fT_4] in people about 10,000:1 but only about 1000:1 in dogs and cats (see the units used for [tT_4] and [fT_4]).

B. T_4 is generally regarded as a prohormone, though it may have direct effects.[20] fT_4 enters most cells of the body and is converted to either T_3 or reverse-T_3 by the actions of selenium-containing deiodinase enzymes, iodothyronine deiodinase I, II, and III.[3] Reverse-T_3, formed via iodothyronine III, is biologically insignificant, whereas fT_3 is responsible for most or all thyroid hormone activity. Gene polymorphisms and variations in the tissue distribution and activities of these enzymes affect fT_3 availability and therefore thyroidal influence.

C. As with T_4, most plasma T_3 (99 %) is protein bound (specific globulins and albumin).

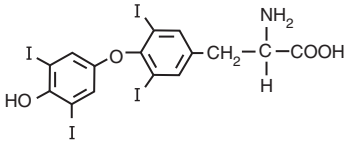
III. T_3 and T_4 dissociate from plasma proteins and enter cells via any of several transporters; within cells, they exert most of their effects by binding to nuclear receptor proteins.[21] They also exert some nongenomic effects on cell signaling pathways by binding to cell membrane and cytosolic receptors. The hormone-receptor complexes initiate changes to cause or promote increased metabolic rate, increased O_2 consumption, increased heart rate and contractility, enhanced response to catecholamines, bone formation and resorption, increased carbohydrate metabolism, increased catabolism of muscle and adipose tissue, protein synthesis, erythropoiesis, and alterations in lipoprotein metabolism.

IV. T_3 and T_4 are excreted in bile and urine.

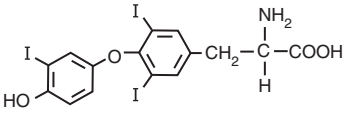
ANALYTICAL CONCEPTS

I. Terms and units

A. Thyroxine (tetraiodothyronine) and triiodothyronine are commonly referred to as T_4 and T_3, respectively. It is important to differentiate tT_4 from fT_4.



Thyroxine



Triiodothyronine

B. Unit conversion[22]

1. tT_4: µg/dL × 10 = ng/mL; µg/dL × 12.87 = nmol/L; ng/mL × 1.287 = nmol/L (SI unit)
2. fT_4: ng/dL × 12.87 = pmol/L (SI unit)
3. T_3: ng/dL × 10 = pg/mL; ng/dL × 0.01536 = nmol/L; pg/dL × 15.4 = nmol/L (SI unit)
4. TSH: µg/dL × 10 = ng/mL, and also reported as mIU/L (or µIU/mL); conversion of ng/mL (or µg/dL) to µIU/mL or the SI unit mIU/L has been done using empirical formulas generated from comparison studies, but the relationship between mass concentration and activity varies with the assay and assay calibration standards, as well as with variations in TSH glycosylation

C. TgAA results may be reported as positive, negative, inconclusive, or as the percentage of a known positive sample.

II. Sample

A. Although either plasma or serum can be used for thyroid hormone testing with some methods, EDTA may interfere with certain methods used for [tT_4], [fT_4], and [TSH]. When using a chemiluminescent enzyme immunoassay in which ALP dephosphorylates a substrate to emit light (e.g., Immulite 1000 and 2000 analyzers), EDTA may falsely decrease [TSH] and falsely increase [tT_4] and [fT_4] by clinically significant magnitudes.[23,24] EDTA chelates divalent cation cofactors (Zn^{2+}, Mg^{2+}) for ALP, and this likely causes or contributes to inaccuracies. The direction of the error is not uniform because assays for tT_4 and fT_4 are competitive and the assay for TSH is not.

1. For the T_4 assays, an increased sample [T_4] decreases the solid-phase binding of ALP-conjugated T_4 reagent, thus leading to less luminescence when ALP cleaves its substrate; luminescence is inversely related to [T_4]. EDTA-induced inhibition of ALP would further decrease light emission, thus causing a false increase in [T_4].
2. In contrast, the TSH immunoassay is not competitive, and a decrease in [TSH] causes a decrease in light emission (and vice versa). Therefore, inhibition by EDTA falsely decreases [TSH].
3. For any of the three assays, underfilled EDTA tubes yield greater inaccuracies because of the resulting greater concentrations of EDTA in the plasma sample.

B. Canine [tT_4] is stable in EDTA-plasma and serum when stored in plastic tubes for as many as 5 d at 22–25 °C or 4 °C, and for months at −20 °C; [tT_4] increased in serum samples stored at 37 °C for 5 d but remained stable in EDTA-plasma samples.[25] Stability of canine [tT_4] (serum or plasma) is similar in glass tubes at 22 °C and −20 °C for 5 d, but [tT_4]

increased when stored in glass tubes at 37 °C, for reasons that are not established. Similar stability is expected in sera from other species.

C. Stability of [fT_4] is similar to that described for [tT_4].[25] As for [tT_4], [fT_4] had increased in both EDTA-plasma and serum samples stored in glass at 37 °C for 5 d.

D. TSH and TgAA are stable at 4 °C for a few days, and for months if frozen.

E. To reach reliable conclusions about the endogenous thyroid status of an animal receiving thyroid medication, a withdrawal period is required to normalize T_4 production after treatment-induced suppression. The required duration of withdrawal likely depends on the dosing regimen and length of treatment; 1 wk was sufficient for euthyroid dogs receiving 26 μg/kg once a day for 16 wk, but 4 wk may be necessary if receiving larger doses.[26] A withdrawal period of 4 wk has been recommended for horses.[27]

F. If assessing for therapeutic T_4 concentrations in a dog receiving levothyroxine, blood should be collected at least 3 h after drug administration and within 8 h if being treated once a day. For monitoring T_3 treatment, samples should be drawn 2–3 h post-treatment. Therapeutic testing should not be done before 2 wk after initiating treatment or a new dosing regimen.[28]

G. Increased plasma [fatty acid] can result in falsely increased [fT_4] in people; the fatty acids displace T_4 on binding proteins. The increased [fatty acids] and falsely increased [fT_4] have been shown to occur when people were given intravenous or subcutaneous heparin.[29,30]

III. Assay principles and procedures

A. [tT_4]

1. [tT_4] is the concentration of free and bound T_4. Sample T_4 bound to T_4-binding proteins can be chemically dissociated to facilitate detection.
2. RIAs were historically the gold standard for measuring [tT_4], but restrictions related to radioisotopes reduced the use of RIAs and increased the use of enzyme immunoassays (chemiluminescent enzyme immunoassay, enzyme-linked immunosorbent assay, spectrophotometric enzyme-linked immunoassay, bulk acoustic wave enzyme immunoassay). Results from different assays, including commonly used methods, do not always agree,[31–35] and there can be good agreement for [tT_4] in one species but not in another.[36] It is best to use a single assay for serial measurements. Remember, assays may correlate very well but not agree.
   a. Comparisons of results have detected biases large enough to dictate use of different RIs for different assays. When a bias is present, it may be present because of inaccurate calibration (standard) solutions. The biases may be constant (the same amount of error in most samples) or the biases can be proportional (a similar percentage error results in a greater absolute error with higher concentrations). When biases are constant, species-specific correction factors can be used to improve agreement among assays.[37]
   b. Compared to a RIA, one spectrophotometric enzyme-linked immunoassay had a proportional bias of –22 % (median), and the assays disagreed on the presence or absence of hyperthyroxinemia in 14 % of health-screening samples from cats > 9 yr old.[34] This enzyme immunoassay was used as the basis for comparison of an automated point-of-care dry-slide ELISA; there was 97 % agreement in diagnoses using the same RI for both assays, but results differed by up to ± 30 %, and the

test population was restricted to cats referred for treatment of hyperthyroidism (assay performance in cats with a variety of disorders was not assessed).[35] Interpretation of assay performance is difficult when assay comparisons do not include gold standard methods and all relevant patient populations.

c. Substantial differences have been detected when results from commercial RIAs were compared to results from commercial enzyme immunoassays (chemiluminescent assay, chemiluminescent enzyme immunoassay, and enzyme-linked immunosorbent assay).[32,36] Those comparisons may no longer apply because of modifications in both types of assays.

3. $[tT_4]$ in healthy dogs, cats, and horses are much lower than $[tT_4]$ in healthy people. Thus, assays for $[tT_4]$ in these species must have lower detection limits to measure normal and decreased $[tT_4]$ correctly. Assays that have an analytical range appropriate for human $[tT_4]$ should not be used to evaluate canine, feline, or equine $[tT_4]$ without appropriate modification and validation.
4. In typical solid-phase RIA systems, T_4AA in serum cause falsely elevated measured $[tT_4]$. With this method, the patient's T_4 competes with a known amount of radioactive T_4 for a limited number of antibody-binding sites (e.g., in a tube coated with anti-T_4 antibodies). Thus, a greater concentration of patient T_4 results in less bound radioactive T_4, which is the measured endpoint. Tube or well radioactivity inversely relates to $[tT_4]$. If T_4AA are present in a patient's sample, they bind some of the radioactive T_4, which therefore cannot bind to the immobilized anti-T_4 antibodies and are washed from the tube and not detected. This yields a falsely low radioactivity which translates to a greater $[tT_4]$ on the standard dose–response curve, hence a falsely increased $[tT_4]$.
5. In some other assays, T_4AAs falsely decrease $[tT_4]$ if the assay detects all antibody-bound radiolabeled T_4 (bound to either reagent antibody or autoantibody). T_4AAs did not affect results for $[tT_4]$ in canine samples tested with one chemiluminescent assay.[38]
6. TgAA, in general, do not interfere with these assays, but sera that contain TgAA may contain T_4AA that do interfere.[39,40]

B. $[fT_4]$

1. Because fT_4 is thought to reflect the available pool of T_4 that can be converted to functional fT_3, accurate measurement of $[fT_4]$ has been a goal. However, its presence at only approximately $1/1000^{th}$ of the concentration of tT_4 makes this difficult. If the ratio of bound to free T_4 remained constant in health and disease, $[fT_4]$ would be proportional to $[tT_4]$, and $[fT_4]$ would not need to be measured. However, disease and drugs can alter fT_4-binding protein concentrations or their affinities for fT_4.
2. Similar to measurement of $[tT_4]$, various competitive enzyme immunoassays have been developed to measure $[fT_4]$. In general, patient fT_4 competes with a reagent T_4 or T_4 analog for an anti-T_4 antibody, with (i) either the T_4 (T_4 analog) or the anti-T_4 antibody labeled for detection, (ii) either the anti-T_4 antibody or the T_4 analog immobilized to a surface prior to washing and signal detection, and (iii) one or two incubations and wash steps. Consequently, assays may be referred to as analog assays or one-step versus two-step assays.
3. Equilibrium dialysis
   a. $[fT_4]$ is best measured by an assay that involves equilibrium dialysis. In equilibrium dialysis procedures, fT_4 is allowed to diffuse through a membrane that does not allow diffusion of protein-bound T_4. After sufficient time has passed, $[T_4]$ is measured in the protein-poor dialysate, or ratios between predialysis and postdialysis values are used to determine a sample's $[fT_4]$.[41]

b. T_4AA do not interfere with the equilibrium dialysis assay because the antibodies do not diffuse through the semipermeable membrane.
c. The major disadvantages of determining $[fT_4]_{ed}$ are the expense and limited availability.
d. Measurement of $[fT_4]_{ed}$ is particularly useful when (i) differentiating hypothyroidism from NTI in dogs with low $[tT_4]$ or $[fT_4]$ but a [TSH] WRI, (ii) T_4AA are present and interfering with accurate assessment of tT_4 and fT_4, or (iii) a patient is receiving medications known to falsely decrease $[tT_4]$ or $[fT_4]$, e.g., glucocorticoids, phenobarbital, or phenylbutazone.

4. Nondialysis fT_4 RIAs
a. Nondialysis RIAs designed for quantitation of human $[fT_4]$ tend to underestimate canine $[fT_4]$. In a comparison of an equilibrium dialysis fT_4 assay and five nondialysis fT_4 assays, the five nondialysis assays yielded 25–85 % lower $[fT_4]$ in sera of healthy dogs and 52–90 % lower $[fT_4]$ in sera of sick dogs with NTI.[41]
b. In a comparison study of the Nichols equilibrium dialysis assay and nine nondialysis assays using sera from euthyroid people, the concordance percentages varied from 59 % to 96 % when serum T_4-binding capacity was WRI, from 66 % to 90 % when serum T_4-binding capacity increased, but from only 31 % to 69 % when serum T_4-binding capacity was decreased.[42] As shown in bias plots, poor agreements were found when the serum T_4-binding capacity was decreased, as may occur with NTI.
c. The efficacy of fT_4 separation from protein-bound T_4 by centrifugation through protein-exclusion membranes varies with the membrane and time of centrifugation, causing inaccurate results in some systems.[43,44] Sample dilution may affect assay biases related to various concentrations of T_4-binding proteins.[45,46]

5. Chemiluminescence assays (no dialysis)
a. When samples from 74 dogs with NTI were assessed for $[tT_4]$ and $[fT_4]$ by a common chemiluminescent analyzer (one-step analog method without dialysis) and compared to RIA results for $[fT_4]_{ed}$, there were 14 discordant fT4 results, all with low $[fT_4]$ but $[fT_4]_{ed}$ WRI, and the $[fT_4]$ largely mirrored the $[tT_4]$: 57 % of the dogs had decreased $[tT_4]$, 53 % had decreased $[fT_4]$, and 34 % had decreased $[fT_4]_{ed}$.[47] In 13 of the 14 discordant cases, $[tT_4]$ was decreased. Unless long-term storage at –80 °C affected results or RIs were inappropriate, these findings indicate disagreement between assays and suggest that $[fT_4]_{ed}$ differentiates NTI from hypothyroidism better than $[fT_4]$ measured with this method.
b. Results from another chemiluminescence assay for $[fT_4]$ were lower than results from an equilibrium dialysis assay. They were about 50 % lower for cat sera.[48] The degree of difference was not reported for dog sera.[49]
c. In the presence of TgAA, a nondialysis method may not detect decreased $[fT_4]$. With the commonly used Immulite (Siemens) one-step analog method, patient fT_4 competes with ALP-labeled T_4 analog for a limited number of anti-T_4 capture monoclonal antibody binding sites on polystyrene beads. With more sample fT_4, less-labeled analog binds the beads, and that leads to less chemiluminescence. If TgAA are in the sample, they may bind the ALP-labeled T_4 analog so that less of it binds to the capture antibodies on the beads, thus creating less chemiluminescence and a falsely increased $[fT_4]$. In a study of 13 hypothyroid dogs that were positive for TgAA, $[fT_4]_{ed}$ was decreased in all of them (mean = 0.2 ng/dL; RI 0.7–2.6 ng/dL), but when 8 of the 13 samples were assayed by a veterinary chemiluminescence

assay for [fT_4], one sample result (2.3 ng/dL) was within the assay's RI (0.7–2.5 ng/dL), one sample result was 16.5 ng/dL, and six sample results were decreased.[50] Therefore, decreased [fT_4] was missed in 25 % of the eight tested samples with TgAA.

6. One must consult assay-specific information to determine if there any significant effects of lipemia or hemolysis, but lipemia may falsely increase $[fT_4]_{ed}$.

C. [tT_3] and [fT_3]
   1. Generally, the same basic principles for T_4 assays (tT_4 and fT_4) apply to T_3 (tT_3 and fT_3) assays. Unlike tT_4 assays, assays designed for measuring [tT_3] in human sera have adequate analytical ranges to measure [tT_3] in dogs and cats.
   2. Immunoassays have the analytical specificity to differentiate T_4 and T_3 molecules. Because serum [tT_3] is much lower than serum [tT_4], assays that measure [tT_3] tend to be less reproducible than assays that measure [tT_4].
   3. Lipemia may affect results (e.g., false increases); assay-specific information should be used to interpret results from lipemic samples.

D. TSH
   1. Canine TSH
      a. There are commercial assays (immunoradiometric and chemiluminescent immunoassays) for canine [TSH], but it is not known whether they detect all glycosylated forms of TSH.[51] The TSH assays lack sufficient analytical range to enable documentation of decreased [TSH] in dogs and cats.[52,53] There was good agreement between two immunoassays when the measured [TSH] was $\geq$ 0.5 ng/mL and fair agreement when the [TSH] was 0.1–0.5 ng/mL.[52]
      b. There is sufficient antigenic variation between human and canine TSH molecules that human TSH assays may not measure canine [TSH] accurately.[54]
      c. The presence of T_3AA or T_4AA does not interfere with TSH measurement.
   2. Feline TSH
      a. Hypothyroidism is an uncommon disorder in cats, and thus there is little need to measure feline [TSH] for hypothyroidism. However, results from a human TSH assay indicated that TSH concentrations in a group of hypothyroid kittens were, as expected with primary hypothyroidism, at least twice the concentration found in healthy cats.[55] Measurement of [TSH] with [tT_4] or [fT_4] may improve detection of mild hyperthyroidism in cats.[53]
      b. A canine TSH assay has been used to provide a canine-equivalent [TSH] in cat sera at measurable concentrations (it cannot detect decreased concentrations).[53]
      c. In an attempt to develop a feline TSH assay, it was found that about 40 % of a recombinant form of feline TSH was detected by canine TSH assays, thus indicating different immunoreactivity of canine and the recombinant feline TSH molecules.[56]
   3. Equine TSH: A RIA for equine TSH was developed by using a noncommercial source of anti-TSH antibody as the ligand, and purified equine TSH as the standard, but a commercial assay has not been available.[57]
   4. Bovine TSH: A RIA for bovine TSH was developed using purified bovine TSH as the standard, the tracer, and the immunogen for generating polyclonal rabbit antibodies to bovine TSH.[58]

E. TgAA
   1. A commercial enzyme immunoassay (EIA) for TgAA (immunoglobulin G, IgG) was developed during the 1990s (produced by Oxford Biomedical Research, now Oxford Laboratories). Prior to its availability, there was a lack of assay standardization. Thus, results from different laboratories were difficult to interpret. Another EIA for

TgAA was subsequently developed, and its results were in good agreement with the commercial assay.[59]

2. For the commercial assay, patient serum is incubated in wells coated with canine thyroglobulin, and an enzyme-conjugated antibody and substrate are used to detect the bound IgG by optical absorbance. Because IgG can bind nonspecifically to the wells, nonspecific binding to uncoated wells is best assessed simultaneously, in which case the difference in absorbance between test and control wells may be used to indicate specific binding. Alternatively, nonspecific binding can be assessed later for only those samples that might be positive. A known positive sample is tested concurrently, and TgAA results are reported as a percentage of the result obtained for the known positive sample. Percentages above a certain decision threshold (e.g., > 25 % or 35 %) are considered positive for TgAA, and another cutoff is used to determine if samples are negative or inconclusive. Prior to 2005, a positive result was an optical density value greater than two standard deviations above the optical density value in a negative control serum.

F. T_4AA and T_3AA: Assays for T_4AA and T_3AA have been used in specialized laboratories for many years. The diagnostic sensitivity of the TgAA values for canine lymphocytic thyroiditis is greater than diagnostic sensitivity values of T_4AA and T_3AA,[39,59] but the latter two results help identify false T_3 and T_4 values that can be caused by interference from T_4AA and T_3AA when using some assays. Lipemia may falsely increase T_3AA results.

G. TpAA: Canine TpAA have been detected in sera by using an immunoblot assay.[40]

THYROXINE (T_4) CONCENTRATION

I. [Total thyroxine] ([tT_4])

A. Hyperthyroxinemia (Table 17.2)

1. Increased production of T_4

a. Thyroid adenoma or adenomatous hyperplasia in cats (thyroid nodules in hyperthyroid cats may be diagnosed as adenomatous hyperplasia but behave like neoplasms, not hyperplasia, because they function autonomously rather than in response to TSH)

Table 17.2. Disorders and conditions that cause hyperthyroxinemia (increased [tT_4])

Increased production of T_4
Thyroid adenoma or adenomatous hyperplasia* (common in cats, uncommon in dogs, rare in horses)
Thyroid adenocarcinoma (dogs, cats, horses)
Ingestion of a large amount of T_4 (food, treats, animal parts containing thyroid tissue, feces of thyroxine-treated individuals)
Administration of T_4, TSH, or TRH
Administration of compounds containing iodide
Selenium deficiency (ruminants)
Positive interference by T_4AA in some tT_4 assays
Neonates (if results are compared to adult RIs)

* A relatively common disorder, condition, or mechanism

Note: Physiologic factors (e.g., breed differences, age, pregnancy, lactation, season, time of day) should be considered when interpreting [tT_4] (see the text).

(1) When presented with the clinical signs of hyperthyroidism, about 90 % of hyperthyroid cats have increased [tT_4].[60] The most common presenting problems include weight loss and polyphagia. Others include alopecia, polyuria, polydipsia, and nonspecific gastrointestinal or nervous system findings. Laboratory data may include the following: erythrocytosis, azotemia, hyperglycemia, decreased [fructosamine], and increased serum activities of alanine transaminase (ALT), ALP, or creatine kinase (CK).

(2) In hyperthyroid cats, [tT_4] may fluctuate enough to be WRI for a random sample.[61] Also, NTI may lower the [tT_4] in hyperthyroid cats so that the [tT_4] is WRI.[62] If a cat has clinical findings suggestive of hyperthyroidism despite a [tT_4] WRI, and particularly if the cat is ≥ 7 yr of age with a [tT_4] in the upper 15–20 % of the RI,[63] further testing may provide evidence of hyperthyroidism: (i) increased [fT_4], (ii) a [TSH] that is not near or below the detection limit, (iii) a subsequently increased [tT_4], or (iv) inadequate T_3-induced suppression of [tT_4].

(3) Mutations causing constitutive TSH receptor activation and thyroid hormone production have been identified in people with hyperthyroidism, and mutations in the TSH receptor and in stimulatory G proteins involved in transduction of the TSH signal have been identified in cats.[60] They may form secondary to hyperplasia. Although they are inconsistent and have not been linked to causing thyroid gland hyperactivity, G-protein expression and in vitro functional studies of thyroid membranes from hyperthyroid cats suggest that decreased function of inhibitory G proteins may play a role in feline hyperthyroidism.[60,64]

(4) Thyroid adenocarcinomas have been reported to occur in about 2 % of hyperthyroid cats.[65] However, the prevalence of suspected adenocarcinomas and severe hyperthyroidism increased in cats that had thyroidal neoplasia for 4–6 yr, despite treatment with methimazole, which inhibits T_4 secretion but not progression of a neoplasm.[66]

b. Thyroid adenoma or adenocarcinoma in dogs: As many as 25 % of canine thyroid neoplasms may produce sufficient T_4 to cause hyperthyroxinemia and clinical signs of hyperthyroidism. These functional neoplasms can be located anywhere from the base of the heart to the base of the tongue.[67] Clinical signs may include polyuria, polydipsia, weight loss, or a cervical mass and associated problems.[68] As in hyperthyroid cats, serum activities of alanine aminotransferase (ALT) and ALP may be increased.

c. Thyroid adenoma or adenocarcinoma in horses: Hyperthyroxinemia is rarely reported in horses with thyroid neoplasms, most of which are not functional. In one horse with a thyroid adenoma, the [tT_4] was about double the upper reference limit and did not suppress appropriately with a T_3 suppression test.[69]

2. Ingestion of a large amount of T_4
   a. Eating food, treats, or animal parts containing thyroid tissue;[70–72] concurrently increased serum [iodine] supports consumption of food containing iodide-rich thyroid tissue[73]
   b. Coprophagia by a dog living with a levothyroxine-treated dog[74]
3. Administration of T_4, TSH, or TRH
4. Administration of compounds containing iodide: For example, horses may have a transient increase in [tT_4] because of increased production of T_4 after exposure to iodide in supplements containing kelp, or in expectorants, counterirritants, contrast media, leg paints, or shampoos.

5. Selenium deficiency (ruminants) impairs iodothyronine deiodinase selenoenzyme activities, and therefore the formation of T_3 from T_4 in tissues, promoting increased [tT_4]. Decreased [fT_3], responsively increased [TSH], and a decreased T_3 to T_4 ratio are expected and were present in lambs with selenium-deficiency myopathy, sometimes with mild increases in [tT_4].[75,76] In contrast, serum selenium concentration was positively associated with [tT4] in a study of selenium deficiency in horses.[77]
6. T_4AA may cause positive interference (false increases) in some tT_4 assays.[37]
7. Age differences: In general, [tT_4] is greatest in neonates and decreases with age. The [tT_4] in 1- to 6-wk-old nursing puppies was 50 % greater than in 12- to 52-wk-old dogs, and [tT_4] decreased with age such that 6- to 11-yr-old dogs had 25 % lower values than 1-yr-old dogs.[9] In 5- to 6-wk-old kittens, the [tT_4] is reportedly 2–3× that of adult cats.[78] Neonatal foals have a much greater [tT_4] than adult horses; [tT_4] decreases to an adult RI in 2–4 wk (mean [TSH] was within the adult RI during those weeks); a similar trend is seen in [tT_3], [fT_3], and [fT_4]).[79] Differences also occur in adult horses, with [tT_4] being lower and [TSH] being higher in older adults.[80] Calves have a greater [tT_4] than adult cattle for the first three weeks, particularly in the first week.[81] In llama crias, the [tT_4] is greatest at birth and decreases to about one-fourth the concentration by 1–3 mo of age.[82] The [tT_3] is also greatest for about the first week of life, and decreases to about one-third the concentration by 1–3 mo of age.

B. Hypothyroxinemia (Table 17.3)
   1. Decreased production of T_4

Table 17.3. Disorders and conditions that cause hypothyroxinemia (decreased [tT_4])

Decreased production of T_4
Acquired primary hypothyroidism:* lymphocytic thyroiditis, idiopathic thyroid atrophy, polyglandular endocrinopathy, destruction of thyroid gland (neoplasms, surgery, radioactive iodide treatments, or other means), iodine deficiency or excess, ingestion of T_3-rich food (thyroid contaminated)
Congenital primary hypothyroidism: thyroid gland dysgenesis, dyshormonogenesis, in utero iodine deficiency or excess
Secondary hypothyroidism: TSH deficiency caused by pituitary malformation or destruction by neoplasms, radiation, or other means
Tertiary hypothyroidism: TRH deficiency caused by a hypothalamic lesion
Multifactorial (may include decreased T_4 production) or unknown mechanisms
NTI:* hyperadrenocorticism, inflammatory diseases
Drugs:* glucocorticoids, trimethoprim-sulfadiazine, trimethoprim-sulfamethoxazole, phenobarbital, phenylbutazone, acetylsalicylic acid, clomipramine
Diets high in energy, protein, copper, or zinc (horses)
Food deprivation for as little as 4 d (horses)
Congenital hypothyroidism and dysmaturity syndrome (foals)
Syndrome of poor growth, angular limb deformities, and anemia in young llamas
Diet high in *Leucaena leucocephala* or fluoride (cattle)

* A relatively common disorder, condition, or mechanism

Note: Physiologic factors (e.g., breed differences, age, pregnancy, lactation, season, time of day) should be considered when interpreting [tT_4] (see the text). Breed-specific RIs would be helpful when available for certain breeds (e.g., sighthound breeds).

a. Acquired primary hypothyroidism (thyroid gland disease): Spontaneously acquired primary hypothyroidism is a common disorder in dogs but rare in cats and other domestic mammals.[83] The most common presenting problems in dogs include weight gain, lethargy, and symmetric alopecia; the more common abnormal laboratory data include mild anemia (nonregenerative), codocytosis, hypercholesterolemia, and hypertriglyceridemia. Clinical abnormalities in cats include a palpable goiter, polyuria and polydipsia, or signs of hypothyroidism, namely hair loss, lethargy, and obesity; the most common reported laboratory abnormalities are azotemia and mild anemia. A basal [tT_4] is the standard screening test for hypothyroidism in dogs; decreased values support hypothyroidism, but there are several reasons for hypothyroxinemia other than hypothyroidism. Conversely, it is very unlikely that a dog with a [tT_4] that is WRI has primary hypothyroidism (if the [tT_4] is valid). Decreased [fT_4]$_{ed}$ and increased [TSH] would further support hypothyroidism. Expected findings in cats with primary hypothyroidism (rare) are similarly decreased [tT_4], decreased [fT_4], and increased [TSH].
   (1) Lymphocytic thyroiditis, considered an inheritable autoimmune disorder in many dog breeds, is a common cause of thyroid gland destruction and primary hypothyroidism in dogs (at least 50 % of cases).[84] The damaged thyroid gland exposes antigens from immunologically privileged sites so that the immune system produces autoantibodies such as TgAA, T_4AA, T_3AA, and TpAA.
   (2) Idiopathic thyroid gland atrophy is another common finding in dogs with hypothyroidism. It may be the terminal stages of lymphocytic thyroiditis.
   (3) Autoimmune hypothyroidism as part of a polyglandular endocrinopathy (polyglandular autoimmune syndrome) has been rarely reported in dogs.[85]
   (4) Thyroid neoplasms may destroy enough functional follicular cells to create a hypothyroid state. Conversely, old, untreated, hypothyroid beagles had a relatively high incidence of thyroid neoplasms compared to old euthyroid beagles, possibly because of chronic TSH stimulation.[86]
   (5) Thyroid glands can be damaged during surgery, during radioactive iodide treatments, and by other physical means. Animals treated for hyperthyroidism may have sufficient thyroid gland destruction to create hypothyroidism. Of 38 cats with hypothyroxinemia after methimazole treatment, 26 had increased [TSH] (measured with the canine TSH assay) and 30 cats had decreased [fT_4]$_{ed}$.[87] Of 189 hyperthyroid cats treated with standard-dose ^{131}I (4 mCi, n = 39) or low-dose ^{131}I (2 mCi, n = 150), about two-thirds of the standard-dose cats developed subclinical or overt hypothyroidism with increased [TSH] compared to about one-fifth of the low-dose cats.[88] Of 50 dogs with available data after surgical resection of functional thyroid adenocarcinomas or adenomas, 32 (64%) were hypothyroid based on decreased [tT_4] or [fT_4].[68]
   (6) Iodine deficiency: Insufficient dietary iodine impairs T_4 synthesis and cause hypothyroidism and goiters as reported in foals.[89] The tT_4 concentrations in affected foals were similar to those in adults but lower than those in age-matched control foals; affected foals also had decreased tT_4:tT_3 ratios. Restricted iodine intake can reduce [tT_4] in hyperthyroid cats.[90]
   (7) Iodine excess: Excessive iodine exposure can cause either hypothyroid or hyperthyroid states depending on underlying factors including pre-existing goiter, iodine deficiency, thyroiditis, and stage of development (fetus, neonate, adult).[91] Usually, in health, increased iodine intake causes a transient increase in

T_4 production followed quickly by an inhibition of production, likely mediated by iodinated molecules, and then escape from inhibition within a few days.[91] Hypothyroidism can occur if escape from inhibition does not occur. Young dogs (45–90 d) on a diet containing excessive iodine developed hypothyroidism with increased [TSH], decreased [tT_4] and [fT_4], and altered skeletal development.[92,93]

(8) Ingestion of T_3-rich, thyroid-contaminated food (e.g., canned food containing beef or bison) may cause thyrotoxicosis with increased [T_3], low [TSH] (expected from T_3-induced suppression of TSH production), and decreased [tT_4] and [fT_4].[73] Finding an increased serum [iodine] may help support that there had been increased consumption of iodide-rich thyroid tissue.

b. Congenital primary hypothyroidism, either hereditary or nonhereditary (acquired in utero)

(1) Congenital primary hypothyroidism caused by thyroid gland dysgenesis (defective thyroid gland development) has been reported in dogs[94] and cats.[95] Congenital nongoitrous hypothyroidism found in two Scottish deerhound puppies may have been caused either by primary thyroid hypoplasia or by thyroid-unresponsiveness to TSH.[96]

(2) Congenital primary hypothyroidism caused by dyshormonogenesis (defective hormone synthesis) has been reported in dogs, cats, and horses. It is commonly associated with enlarged thyroid glands (goiters) in response to persistent TSH stimulation.

(a) In dogs, defective iodine incorporation into thyroglobulin (iodine organification defect) has been documented secondary to (i) a mutation in the gene encoding the sodium/iodine symporter involved in iodine uptake by follicular cells (shih tzus),[97] and (ii) impaired iodide oxidation because of congenital thyroid peroxidase functional deficiency in Spanish water dogs, a French bulldog, and rat, toy fox, or Tenterfield terriers.[98,99] The shih tzus with a defect in iodine transport lacked the mental dullness and disproportionate dwarfism seen in the other dogs with congenital hypothyroidism.

(b) In cats, congenital hypothyroidism and dyshormonogenesis has been associated with a missense mutation in the gene for thyroid peroxidase; this was documented in Russian blue, British shorthair, and domestic short-, medium-, and long-hair cats, and has a recessive mode of inheritance.[100] The condition is characterized by disproportionate dwarfism, decreased to low-normal [tT4], increased [TSH], and decreased thyroid accumulation of ^{123}I (based on scintigraphy) because of defective iodine organification. Dyshormonogenesis has been suspected as the cause of congenital hypothyroidism in other hypothyroid kittens with dwarfism and enlarged thyroid glands,[101] and rarely in adult cats.[102] The cause has been unclear in several other reports of congenital hypothyroidism.[55,103] Note: Because healthy 5- to 6-wk-old kittens have tT_4 concentrations that are 2–3 times adult cat concentrations, a hypothyroid kitten may have a [tT_4] within the adult RI.[78]

(3) Iodine deficiency or excess while in utero: In calves, hypothyroidism and congenital goiters have been associated with iodine deficiency from decreased dietary intake of iodine by the dam during gestation.[81,104] This could occur in other species as well, and a pregnant dam's ingestion of goitrogenic plants could

also affect iodine availability. As supported by studies in rabbits, excess iodine ingestion during pregnancy can impair fetal thyroid gland development and cause fetal hypothyroidism that may persist after birth if a fetus survives.[105,106]

c. Secondary hypothyroidism (caused by TSH deficiency) is one form of central hypothyroidism. It may be an isolated TSH deficiency or a combined pituitary hormone deficiency as reported in German shepherd and related dogs (Saarloos and Czechoslovakian wolfdogs) with pituitary dwarfism.[107]
   (1) Lack of TSH secretion causes atrophy of thyroidal follicular cells and thus decreased T_4 and T_3 production, as suspected in giant Schnauzers with congenital hypothyroid dwarfism.[108]
   (2) Rare cases caused by pituitary neoplasms or malformation are reported in dogs and cats. It may also occur because of pituitary gland damage caused by radiation, surgery, or other trauma.[109]
   (3) Thyroid neoplasms may produce abnormal hormones that do not stimulate physiologic responses (therefore hyperthyroidism does not develop) but do suppress TSH release (therefore hypothyroidism develops).[110]

d. Tertiary hypothyroidism (caused by TRH deficiency), another form of central hypothyroidism: A pituitary neoplasm that invaded the hypothalamus was considered the cause of tertiary hypothyroidism in a dog with pituitary-dependent hyperadrenocorticism.[111] The dog had hypothyroxinemia and adequate pituitary and thyroid responses in TRH and TSH response tests.

e. Several dogs have been reported to have central congenital hypothyroidism without complete localization and characterization.[112] Horses with pituitary pars intermedia dysfunction may have a degree of central hypothyroidism given decreased $[fT_4]_{ed}$ without appropriate increases in [TSH]; response to TRH was not different from that in healthy horses.[113]

2. Multifactorial (may include decreased T_4 production) or unknown mechanisms
   a. NTI
      (1) Many inflammatory, neoplastic, metabolic (e.g., renal failure), and endocrine disorders (especially hyperadrenocorticism) are known to decrease $[tT_4]$, more so with increased disease severity. This hypothyroxinemic state created by a disease outside the thyroid hormonal system is called *nonthyroidal illness syndrome*. It has also long been termed *sick euthyroidism* or *sick euthyroid syndrome* (*eu* from Greek, meaning "well").
      (2) The mechanisms that create the hypothyroxinemia involve a degree of central hypothyroidism and may include the following, depending on the illness.[46]
         (a) When illness causes reduced caloric intake, lower leptin concentrations lead to less stimulatory effect of melanocyte-stimulating hormone on TRH expression. Lower TRH production results in less TSH release and thus less T_4 production.
         (b) Inflammatory cytokines promote increased activity of iodothyronine deiodinase in tanycytes, which results in more T_3 production in the hypothalamic area. The T_3 then inhibits TRH production, resulting in less T_4 production.
         (c) Inflammatory cytokines (especially interleukin 6) may directly suppress TSH release from pituitary thyrotropes.
         (d) Thyroxine-binding globulin, transthyretin, and albumin are negative acute-phase proteins. Thus, during inflammation, decreased concentration of these thyroxine-binding proteins can cause a decrease in the $[tT_4]$.

(e) In some conditions such as renal or hepatic disease (as with certain drugs), circulating molecules may compete with T_4 for binding sites on T_4 carrier proteins, thus decreasing [tT_4].

(f) Other factors that could alter [tT_4] include decreased tissue uptake of T_4, altered intracellular T_4 deiodination, and changes in nuclear T_4 receptors or coactivators.

(3) Assessment of TSH and [fT_4]$_{ed}$ may help differentiate NTI from hypothyroidism when [tT_4] is decreased in dogs or cats with suspected hypothyroidism; in the majority of patients with NTI, [fT_4]$_{ed}$ is not decreased and TSH is not increased, although increased [TSH] is not uncommon and [fT_4]$_{ed}$ may be decreased. With recovery from illness, TSH may be transiently increased, thus mimicking primary hypothyroidism.

(4) Horses with more severe illnesses had lower concentrations of tT_4, fT_4, tT_3, and fT_3; [fT_4]$_{ed}$ generally did not decrease as much as [tT_4], but decreases occurred. TSH concentrations typically remained within the RI, consistent with thyroid dysregulation.[114]

b. Several drugs (especially glucocorticoids) are known to lower [tT_4].

(1) Dogs with hyperadrenocorticism frequently have decreased [tT_4],[115] but changes in [tT_4] in dogs receiving glucocorticoid therapy are variable, likely related in part to dose and to duration of exposure. The [tT_4] did not change in one study,[116] whereas the [tT_4] decreased in another study.[6] There is evidence that the decreases in [tT_4] (and [fT_4]) are caused by multiple factors, including reduced TSH secretion[115,117] and decreased thyroid gland responsiveness to TSH.[6] There is also evidence that glucocorticoids reduce the conversion of T_4 to T_3 systemically.[115] Increased [TSH] is not expected.

(2) In dogs, prednisolone,[6] prednisone (daily anti-inflammatory dose),[118] phenobarbital,[119,120] and acetylsalicylic acid (aspirin)[121] treatments have been shown to cause hypothyroxinemia ([tT_4] and [fT_4]$_{ed}$) without clinical hypothyroidism. Trimethoprim-sulfadiazine[122] and trimethoprim-sulfamethoxazole[123] cause hypothyroxinemia by inhibiting thyroid peroxidase activity and therefore iodination of thyroglobulin,[124,125] so [TSH] may be increased and clinical signs of hypothyroidism may develop. Clomipramine (ClomiCalm) administered to dogs for nearly 4 mo decreased [tT_4] and [fT_4]$_{ed}$. The decrease in [tT_4] was detectable after 1 mo of treatment; it persisted for the duration of the experiment, but did not drop below the lower reference limit.[126] Clomipramine is used for a variety of behavioral disorders. Prolonged antineoplastic use of certain tyrosine kinase inhibitors (e.g., toceranib, sunitinib) may cause subclinical or perhaps clinical hypothyroidism in dogs, as it does in people.[127]

(3) In horses, phenylbutazone and glucocorticoid treatments (including topical) have been shown to cause hypothyroxinemia;[128] phenylbutazone decreased [tT_4] and [fT_4] but did not impair response to TSH.[129,130]

(4) Mechanisms by which these and other drugs create hypothyroxinemia include (i) decreasing TSH secretion, (ii) decreasing thyroid hormone secretion, (iii) altering T_4 transport in plasma, and (iv) increasing the hepatic catabolism of tT_4.[131]

c. The following have been shown to produce lower [tT_4] in horses:

(1) Diets high in energy, protein, copper, or zinc

(2) Food deprivation for 4 d (mean [tT_4] decreased from 19.9 nmol/L to 7.6 nmol/L)[132]

d. Congenital hypothyroidism and dysmaturity syndrome: Foals are born with thyroid hyperplasia, skeletal abnormalities, and inappropriately immature features for neonates.[133,134] It is generally diagnosed by clinical findings and histologic evaluation of the thyroid gland, but some of the foals have decreased [tT4] for their ages, from as early as 1 d to several months of age.[133] The cause is unclear but may involve ingestion of nitrate-containing iodine-deficient diets, goitrogenic plants, or insufficient selenium by mares during gestation.[135] Nitrate has been reported to compete with iodine for thyroidal uptake by the sodium/iodide symporter, and selenium is a component of the deiodinases that convert T_4 to T_3 in the thyroid gland and other tissues.

e. Hypothyroxinemia has been reported in young llamas with a syndrome characterized by poor growth, angular limb deformities, and anemia. In TSH stimulation testing, the tT_4 concentrations were less than RIs whereas the tT_3 concentrations were variable (low, within, or high). Thyroid hormone supplementation increased tT_4 and tT_3 concentrations but was not associated with clinical improvements.[136]

f. Ruminants fed foliage from *Leucaena leucocephala* (common names: miracle tree, subabul, ipil-ipil, uazin, yaje, and kubabule) developed hypothyroxinemia and hypothyroidism.[137]

g. Dairy cattle suffering from fluorosis (excess fluoride via mineral supplements) had reduced tT_4 and tT_3 concentrations that were inversely related to urine fluoride concentrations; RIs for the hormone concentrations were not reported, nor was evidence of clinical hypothyroidism.[138]

3. Certain dog breeds tend to have lower [tT_4] than other breeds.

a. In one study, the mean [tT_4] in large-breed and medium-breed dogs was about 0.5 μg/dL lower than the [tT_4] in small-breed dogs.[9] However, in another study, size did not explain differences of as much as 0.7 μg/dL in the mean [tT_4] of seven breeds of dogs, with English setters having the lowest mean value and keeshonden the highest.[8] In another study, the [tT_4] interval for Australian basenjis was lower (2–27 nmol/L) than a laboratory's RI (17–37 nmol/L).[10]

b. Greyhounds and other sighthound breeds have lower reference values for [tT_4] and [fT_4] than most other dogs.[11,12] Concentrations of tT_4 and fT_4 were below breed-nonspecific lower reference limits in about 90 % and 20 %, respectively, of young, healthy greyhounds, including values of each below detection limits.[139] RIs derived from results for over 200 healthy Salukis (a sighthound) indicate that tT_4, fT_4, tT_3, and fT_3 concentrations are lower than typical canine RIs, whereas the upper reference limit for [TSH] is higher.[12] Similar lower tT_4 concentrations have been found in sloughis (also [fT_4]) and whippets, which are also sighthounds.[13] The diagnosis of hypothyroidism in sighthounds may require assessment of [TSH]; if [TSH] is WRI in a sighthound with low [tT_4] and [fT_4], the dog is unlikely to be hypothyroid.

4. The pre-race [tT_4] of some endurance-trained Alaskan sled dogs was decreased, and [tT_4] decreased further after an endurance race.[140] The baseline differences from the general population may relate to their training and/or to genetic selection, as these mixed breed dogs have as much genetic distinctiveness as American Kennel Club breeds.[141]

5. In dogs, there is a progressive decrease in [tT_4] and TSH responsiveness with age such that the [tT_4] in older dogs is likely to be in the lower part of the RI.[142] Older horses also tend to have lower [tT_4] but higher [TSH] than younger horses.[80]

II. [Free thyroxine]$_{ed}$ ([fT_4]$_{ed}$) (fT4 Concentration by Equilibrium Dialysis)
  A. Increased [fT_4]$_{ed}$
    1. The same disorders that cause a truly increased [tT_4] (Table 17.2) are expected to cause a concurrently increased [fT_4]$_{ed}$. About 98 % of hyperthyroid cats have increased [fT_4]$_{ed}$ when tested,[143] but increased [fT_4]$_{ed}$ is not specific for feline hyperthyroidism.
    2. [fT_4]$_{ed}$ does not always mirror [tT_4].
      a. Concurrently finding decreased [tT_4] and increased [fT_4]$_{ed}$ in cats suggests the presence of a NTI (NTI syndrome, sick euthyroid syndrome). In about 90 % of cats with NTI, [fT_4]$_{ed}$ is WRI, but it is increased (usually mildly) in about 6 % of them.[144] The cause of the increases is unknown. The accompanying [tT_4] is usually less than the midpoint of the [tT_4] RI. In contrast, a [tT_4] in the upper half of the RI in a cat with increased [fT_4] suggests hyperthyroidism.[145] Lack of increased [tT_4] in hyperthyroid cats may reflect early disease, fluctuations in [tT_4], or suppressive effects of concurrent disease.
      b. A horse with clinical hyperthyroidism had a normal [tT_4] but an increased [fT_4]$_{ed}$.[146]
    3. T_4AA may cause positive interference in some fT_4 assays,[50] but values for [fT_4]$_{ed}$ are not affected.
  B. Decreased [fT_4]$_{ed}$
    1. Thyroidal disorders that cause decreased [tT_4] by decreasing production of T_4 are expected to have concurrently decreased [fT_4]$_{ed}$.
    2. Other disorders or conditions that may cause decreased [fT_4]$_{ed}$ include hyperadrenocorticism, inflammation (interleukin 1, interleukin 2, interferon γ, and tumor necrosis factor may cause decreased [fT_4]$_{ed}$), and treatments with glucocorticoids, sulfonamides, phenobarbital, clomipramine, or high-dose aspirin.[121]
    3. Decreased [fT_4]$_{ed}$ was found in horses with experimentally induced hypothyroidism and in horses with a variety of illnesses (especially with severe disorders).[114,147]

TOTAL TRIIODOTHYRONINE (tT_3) AND FREE TRIIODOTHYRONINE (fT_3) CONCENTRATIONS

I. Basal [tT_3] and [fT_3] assess conversion of T_4 to T_3 but have less diagnostic sensitivity for hypothyroidism and provide little useful additional information over [tT_4] and [fT_4]. Serum [tT_3] is often decreased in dogs and cats with NTI, sometimes below detection limits.[144,148] In dogs, diurnal variations in [tT_3] were less than the variations found in [tT_4].[5]

II. [tT_3] is measured to monitor therapeutic concentrations and to assess owner compliance in the T_3 suppression test.

III. If a solid-phase RIA is used to measure [tT_3], then T_3AA can cause erroneously increased values just like T_4AA in tT_4 assays. In some assays, T_3AA falsely decrease [tT_3] and falsely increase [fT_3].

THYROID-STIMULATING HORMONE (TSH) CONCENTRATION

I. Dogs
  A. Increased canine [TSH]
    1. Primary hypothyroidism: Lack of negative feedback because of decreased [fT_4] and decreased [fT_3] should increase pituitary synthesis and release of TSH and thus cause an

increased [TSH]. Studies indicate that about 60–75 % of hypothyroid dogs have increased [TSH].[149–152]

2. Compensated hypothyroidism: It appears that some dogs with thyroiditis can maintain [tT_4] or [tT_3] WRI but only with greater stimulation of remaining thyroid cells by TSH.
3. Eleven of 30 dogs with hypoadrenocorticism had increased [TSH] without hypothyroxinemia; in each, the [TSH] normalized with therapy.[117] All 30 dogs with other disorders mimicking hypoadrenocorticism lacked an increased [TSH], and some had hypothyroxinemia.
4. [TSH] is expected to increase during the TRH response test (see Response and Suppression Tests, Section II).

B. Because [TSH] in euthyroid dogs can be at the detection limit of a canine TSH assay, a decreased [TSH] may not be reliably differentiated from a [TSH] that is WRI. Therefore, [TSH] may be WRI in dogs with secondary (pituitary) hypothyroidism. Hypothyroid dogs without increased [TSH] may have secondary hypothyroidism or primary hypothyroidism, although secondary hypothyroidism is considered much less common. In dogs with thyroidectomy-induced primary hypothyroidism, [TSH] immediately increased but returned toward baseline over 3 yr, to WRI for some dogs.[153] This is consistent with clinical findings of no increase in [TSH] in a minority of dogs with suspected primary hypothyroidism, but the mechanism is not clear.[149–152]

C. Dogs with NTI syndrome or drug-induced changes in thyroidal hormone concentrations also most commonly have [TSH] values that are WRI, but they may be increased.[149,151,152] Lack of appropriate increases in [TSH] in response to decreased [tT_4], [fT_4], or [tT_3] in NTI reflects thyroid hormone dysregulation.[46,148] [TSH] may increase transiently with recovery from NTI.

II. Cats

A. By using a canine TSH RIA to attempt to measure feline TSH, the feline [TSH] in 50 healthy cats was 0.00–0.32 ng/mL (canine TSH was used for standard solutions).[154] If there is not 100 % cross-reactivity between canine and feline TSH, then the use of purified feline TSH in the assay would produce different results. However, the analytical inaccuracy would be reflected in the RI, so interpretations may not be affected. Feline TSH RIs derived for a canine chemiluminescent enzyme immunoassay were similar: < 0.03–0.3 ng/mL (0.03 ng/mL being the lower limit of detection).[53] In this study, [TSH] was appropriately suppressed (undetectable) in 98 % of 917 hyperthyroid cats, but 14 hyperthyroid cats had measurable [TSH], and [TSH] was undetectable in 30 % of 163 euthyroid cats. Therefore, although a low [TSH] with the canine chemiluminescent assay has high diagnostic sensitivity for hyperthyroidism, meaning that a [TSH] not near or below the lower reference limit helps exclude hyperthyroidism in cats, a low [TSH] has low diagnostic specificity for hyperthyroidism.

B. Increased concentrations of [TSH] were found in cats after thyroidectomies, chronic methimazole therapy, or radioiodine therapy for hyperthyroidism (iatrogenic hypothyroidism).[87,88,154–156] Increased [TSH] is also documented in congenital hypothyroidism with goiter caused by dyshormonogenesis.[102]

C. In most cats with NTI, [TSH] is WRI or below detection limits, and more often undetectable (31 %) than in healthy cats (25 %).[144] Increased [TSH] was present in slightly more cats with NTI (3.2 %) than in healthy cats (2.1 %), but both percentages are close to the 2.5 % of mildly increased values expected when RIs reflect the central 95 % of healthy cats. With NTI, [TSH] increases are usually milder than expected with primary hypothyroidism.

D. A new feline immunoassay that uses bulk acoustic wave technology is purported to have a lower limit of quantification of 0.008 ng/mL and to be able to detect decreased [TSH] in cats, but independent peer-reviewed evaluations were not found.[157]

III. Horses: Equine [TSH] was increased in horses after experimental production of hypothyroidism and as a response to TRH administration.[57] Additional studies are needed to assess the diagnostic value of [TSH] in horses. As in other species, NTI causes thyroid dysregulation which typically keeps [TSH] WRI despite decreases in [tT_4], [fT_4], or [tT_3].[114]

IV. Cattle: Bovine [TSH] is greater in newborn calves than in adults and was increased in calves with congenital goiters compared to healthy calves.[58]

AUTOANTIBODIES

I. Thyroglobulin autoantibodies (TgAA)
   A. The reported prevalence of TgAA in dogs with hypothyroidism varies, but the percentages are relatively large: 36 % of 42 dogs[158,159] and 55 % of 31 dogs.[59] With marked thyroid atrophy or end-stage lymphocytic thyroiditis, the amount of circulating TgAA may diminish.[160]
   B. In one study, all eight dogs with a positive TgAA result had lymphocytic thyroiditis.[161] TgAA probably form when thyroglobulin is released from a damaged thyroid gland and the body reacts to the "foreign" protein. A positive TgAA result is evidence of thyroid disease (inflammatory or rarely neoplastic) and not evidence of thyroid dysfunction.
   C. Predictive values of TgAA results for thyroidal disease have varied among investigators, at least partially because of different methods of establishing a decision threshold between positive and negative reactions.[161,162] Positive results were also found in dogs with hypercalcemia, hypocalcemia, hypoadrenocorticism, hyperadrenocorticism, or diabetes mellitus. It was not determined whether these positive reactions were true or false-positive results,[161] but TgAA positivity has been reported in a dog with suspected polyglandular (thyroid and adrenal) endocrinopathy.[85]
   D. When TgAA results are part of a thyroid profile, the data can be used to stage the progression of canine lymphocytic thyroiditis and primary hypothyroidism (Table 17.4).[159]

Table 17.4. Stages of canine lymphocytic thyroiditis and thyroid gland dysfunction[a]

Stage	[tT_4][b]	TgAA	[TSH]	Histologic findings
1	WRI	Positive	WRI	Majority normal with peripheral lymphocytic infiltrate
2	WRI	Positive	↑	Marked lymphocytic thyroiditis
3	↓	Positive	↑	Less than 25 % of normal thyroidal tissue
4	↓	Negative	↑ or WRI	Minimal remaining thyroidal tissue

[a] Modified from published information[159]

[b] For some tT_4 assays, T_4AA may cause a positive interference such that a "WRI" becomes an ↑ result or a "↓" result becomes a WRI result. A valid [fT_4] may be needed to establish the degree of thyroid gland dysfunction.

1. Stage 1: The TgAA status may be positive for many months before there is clinical or other laboratory evidence of thyroid gland dysfunction.[163]
2. Stage 2: [TgAA] remains increased and the [T_4] remains within its RI because of increased TSH, which stimulates increased production of T_4 by the remaining thyroidal tissue (still subclinical).
3. Stage 3: Clinical hypothyroidism is present with classic laboratory findings.
4. Stage 4: Because very little thyroglobulin is released from the remnants of thyroidal tissue, there is less stimulus for TgAA production. [TSH] is expected to be increased but may not be in chronic hypothyroidism. Reasons for the lower [TSH] are not established but might be through NTI mechanisms or perhaps other factors limiting TRH or TSH production.

II. Thyroxine autoantibodies (T_4AA) and triiodothyronine autoantibodies (T_3AA)
   A. Many dogs with lymphocytic thyroiditis also have autoantibodies against T_4 (T_4AA) and, more often, T_3 (T_3AA). These may be TgAA that react with thyroglobulin-bound T_4 and T_3. In a study of nearly 300,000 dogs with clinical signs of hypothyroidism, thyroid hormone autoantibodies were found in about 6 % of the dogs' sera.[160] Of 149 hypothyroid dogs in another study, T_4AAs and T_3AAs were detected in 2.7 % and 9.4 % of the samples, respectively, with none detected in 110 healthy dogs.[38]
   B. When autoantibodies are detected, TgAA are more common than either T_4AA or T_3AA. However, some dogs have a negative TgAA result and a positive T_4AA or T_3AA result.[39,59]
   C. Positivity for T_4AA or T_3AA in a dog is evidence of lymphocytic thyroiditis and potential interference with the corresponding assays for [tT_4], [fT_4] (not [fT_4]$_{ed}$), [tT_3], and [fT_3].

III. Thyroid peroxidase autoantibodies (TpAA)
   A. Thyroid peroxidase catalyzes the iodination of tyrosyl groups of thyroglobulin in thyroidal colloid. The iodination leads to the formation of T_4 and T_3 within thyroglobulin molecules. The hormones are cleaved from the thyroglobulin in thyroid epithelial cells and are released to blood.
   B. The diagnostic value of the canine TpAA assay has not been established. In canine sera that contained TgAA, T_4AA, or T_3AA, 17 % contained TpAA.[40] TpAA may be involved in the pathogenesis of lymphocytic thyroiditis, but there are also other proposed theories for the immune-mediated destruction of thyroid epithelial cells.[164]

RESPONSE AND SUPPRESSION TESTS

I. Thyroid-stimulating hormone (TSH) response test (TSH Stimulation Test)
   A. A TSH response test assesses the ability of the thyroid gland to produce T_4 in response to being stimulated by TSH. This can differentiate hypothyroxinemia of NTI from that of hypothyroidism.
   B. Poor or inadequate response to bovine TSH was considered the gold standard for establishing thyroid hypoplasia (hypothyroidism) for many years, but an approved pharmacologic form of bovine TSH is no longer available. Recombinant human TSH is available and has been used in dogs; it compared favorably to the use of bovine TSH.[165–167] It has also been evaluated in cats.[156,168]
   C. Several protocols have been recommended. The amount of administered TSH and the sample collection times vary among feline, canine, equine, and llama protocols.

D. Canine TSH response test using recombinant human TSH[169,170]
   1. Procedure: After a serum sample for a basal [tT_4] is collected, 75, 100, or 150 µg of TSH is administered intravenously; a second serum sample is collected 6 h after TSH administration. The expected increase in [tT_4] after TSH administration is less if smaller amounts of TSH are given, e.g., 75 µg[169] or 100 µg[167] compared to the more expensive 150 µg.[169]
   2. Interpretation guidelines should vary among tT_4 assays and with the administered dose of TSH, but the lower the post-TSH [tT_4] (values near the lower reference limit), the greater the likelihood of hypothyroidism; the greater the post-TSH [tT_4] (values at least as great as the middle of the RI), the greater the likelihood of euthyroidism.

E. In cats, post-TSH tT_4 concentrations at 6 h were similar with the intravenous administration of 25 µg up to 200 µg human recombinant TSH.[168] Criteria to differentiate euthyroid cats from hypothyroid cats were not described. In another study using 25 µg TSH in six healthy cats, basal [tT_4] was 13–26 nmol/L versus 49–66 nmol/L at 6 h post-TSH, and the RI for [tT_4] was 14–45 nmol/L.[171] Suppressed response to 50 µg of human recombinant TSH was documented in cats treated with radioiodine for hyperthyroidism, whereas cats with noncritical disease and [tT_4] WRI responded similarly to healthy cats.[156]

F. In healthy horses, post-TSH concentrations of tT_4, fT_4, and T_3 have peaked about 2–6 h after intramuscular or intravenous administration of 5 IU of bovine-derived TSH. At 3 and 6 h after intramuscular administration, [tT_4] was about 2–3 × baseline; [T_3] was about 2–5 × baseline at 3 h.[130] With intravenous administration of TSH, [tT_4] and [fT_4] were 3 × baseline and 4 × baseline, respectively, at 3 h post-TSH, and they remained increased at 6 h; [T_3] was at least 5 × baseline at 2 h post-TSH.[129]

G. In healthy llamas, [tT_4] and [tT_3] increased at least 2× and 2.5× at 4 h and 8 h, respectively. Bovine-derived TSH was injected at 3.0 IU/kg of body weight.[172]

H. When used to assess animals with chronic central hypothyroidism, a single TSH response test may fail to yield an increased [tT_4] because of thyroid atrophy, but repeated administration of TSH over a few days can "reactivate" the thyroid gland and cause an increased serum [tT_4] supportive of central rather than primary hypothyroidism.[112]

II. Thyrotropin-releasing hormone (TRH) response test (TRH Stimulation Test)

A. The TRH response test indirectly assesses thyroid function by causing the release of TSH that stimulates release of T_4. Response is routinely assessed by the [tT_4], but [TSH] and [growth hormone] may also be measured.

B. In dogs, the TRH response test is not as good as the TSH response test in detecting hypothyroidism.[152,173] A good response excludes hypothyroidism, but some euthyroid dogs (as classified by the TSH response test and clinical signs) appear to be hypothyroid (i.e., have inadequate increases in [tT_4]) by the TRH response test, which had a positive predictive value of 50 % in one study.[174]
   1. When assessed in 21 dogs with decreased [tT4] but without increased [TSH], a population that could have hypothyroidism or NTI, the dogs with scintigraphic evidence of hypothyroidism could be differentiated from those without hypothyroidism by their diminished percentage increase (< 50 %) in [TSH] and their increased [growth hormone] 45 min after injection.[175]
   2. The cause of suppressed TSH responses in some hypothyroid dogs is not known, but incomplete detection of TSH by current assays is one possibility, and a consequence is

that the TRH response test does not reliably differentiate primary from secondary hypothyroidism.

C. Reagent-grade and compounded forms of protirelin, the tripeptide synthetic analog of TRH, have been used in horses, but mostly to assess for pituitary pars intermedia dysfunction via adrenocorticotropic hormone responses. Serum [tT_4] increased a mean of 60 % in 12 healthy horses 4 h after intravenous injection of 1.2 mg of protirelin at about 9 a.m., and [TSH] was increased at least 3× at 2 h post-injection,[176] at which time [tT_3] is expected to be 3 × baseline.
   1. Similar protocols have yielded similar results, but the baseline [TSH] is greater and the percentage increase in [TSH] is lesser in older adult horses than in younger adult horses.[80]
   2. Sedation of insulin-sensitive mares with detomidine or a combination of detomidine and butorphanol potentiated the early TSH response to TRH, yielding greater TSH concentrations 5 and 10 min post-TRH compared to mares that received saline instead of a sedative.[177] Increases in [TSH] were similar at 20 and 30 min post-TRH, and later samples were not collected.

D. The TRH response test is typically not needed to diagnose hyperthyroidism in cats but has been used when hyperthyroidism is clinically suspected and repeated results for [tT_4] or [fT_4] are equivocal, or when laboratory results suggest hyperthyroidism, but no thyroid nodule is palpable and thyroid scintigraphy is not available or is equivocal.[145,178] It has also been used in a horse diagnosed with hyperthyroidism from a functional thyroid adenoma.[179]
   1. In hyperthyroid cats, TRH is expected to have little or no effect on TSH and [tT_4] (< 50 % increase) because of feedback inhibition on the pituitary gland. Serum [tT_4] in a healthy euthyroid cat is expected to double in the post-TRH sample.
   2. In the horse with a thyroid adenoma, there were no changes in total or free T_4 and T_3 concentrations 2 or 4 h after intravenous administration of 1 mg TRH.[179]
   3. Advantages of the TRH response test over the T_3 suppression test are that it is shorter (hours versus days) and does not require repeated administration of TRH. Disadvantages are the side effects of TRH administration to cats (salivation, vomiting, tachypnea, and defecation).

III. Triiodothyronine (T_3) suppression test

A. Cats
   1. Most cats with clinical hyperthyroidism have hyperthyroxinemia, and thus additional diagnostic testing is not needed. However, some cats with hyperthyroidism have basal [tT_4] or [fT_4] WRI or only slightly increased (equivocal), perhaps because of variable secretion of T_4 from hyperfunctional thyroid glands, a nonthyroidal disease that is lowering [tT_4], or other reasons. For these cats, a T_3 suppression test should differentiate hyperthyroidism (with defective hypothalamus–pituitary–thyroid regulation) from euthyroidism.[180,181]
   2. Basics of the procedure
      a. Collect serum for basal [tT_4] and basal [tT_3], and freeze it for submission with later serum samples.
      b. Owners administer T_3 (liothyronine) per os starting the next morning at 25 µg q8h for seven treatments.

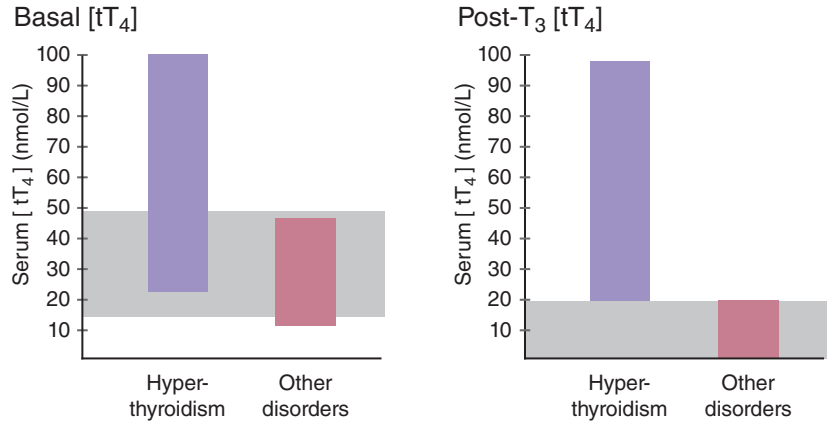


Figure 17.2. T_3 suppression test results in cats; liothyronine, 25 µg per os, q8h for seven doses. The *grey-shaded area* represents the [tT_4] found in 44 clinically healthy cats before and after administration of liothyronine; the lower post-T_3 T_4 concentrations reflect the suppression of TSH release and the subsequent decrease in T_4 production. In cats with hyperthyroidism, there was a failure to suppress [tT_4] below 20 nmol/L. In cats with other disorders, the [tT_4] in post-T_3 samples was < 20 nmol/L. Hyperthyroidism was diagnosed in 77 cats, based on clinical signs, palpable thyroid nodules, high-normal to increased [tT_4], and response to treatment for hyperthyroidism. Cats (n = 22) with other disorders had clinical signs suggestive of hyperthyroidism. Their disorders included gastrointestinal diseases, chronic renal disease, cardiomyopathy, and behavioral disorders. The graph was constructed from published data.[180]

c. Within 2–4 h of the final dose of T_3, the cat is returned to the clinic, and serum is collected for post-T_3 [tT_4] and [tT_3].
d. Basal and post-T_3 sera are submitted to a laboratory – properly labeled at the times of collection – for determination of [tT_4] and [tT_3].

3. Expected results
   a. The post-T_3 [tT_3] should be greater than the basal [tT_3]. If it is not, then failure of suppression may be due to failure to administer T_3 to the cat.
   b. Interpretation guidelines of post-T_3 [tT_4] (Figure 17.2): Values may vary with the assay and laboratory. Using a concentration decision threshold is considered a better method of interpreting results than using a percent decrease,[143,180] but at least a 50 % decrease in [tT_4] typically excludes hyperthyroidism.
      (1) Post-T_3 [tT_4] > 20 nmol/L (> 1.5–1.6 µg/dL) indicates a lack of suppression of T_4 secretion and thus supports the presence of a thyroid adenoma.
      (2) Post-T_3 [tT_4] < 20 nmol/L (< 1.5–1.6 µg/dL) demonstrates a suppression of T_4 secretion and thus indicates that the cat does not have a thyroid adenoma.

B. Horses: Hyperthyroidism is uncommon in horses, and thus the need for a T_3 suppression test is limited. Interpretive guidelines and optimal sampling times are not firmly established.[69] Testing has been done by intramuscular injection of 2.5 mg T_3 in 5 mL saline the evening of day 1 and then twice daily for the next 3 d, with blood drawn 5 min before each injection and additionally twice daily on days 5 and 7 and in the morning

of day 10. Hyperthyroidism is supported by the failure of [T_4] to decrease while [T_3] remains increased.

TOTAL THYROXINE TO THYROID-STIMULATING HORMONE (tT_4:TSH) AND FREE THYROXINE TO THYROID-STIMULATING HORMONE (fT_4:TSH) RATIOS IN DOGS

I. Because of the physiologic relationships among tT_4, fT_4, and TSH secretion, tT_4:TSH and fT_4:TSH ratios have the potential to differentiate hypothyroid from euthyroid states. Lower ratios suggest that the thyroid glands are not responding to endogenous TSH (primary hypothyroidism). However, the tT_4:TSH ratio may also decrease in secondary hypothyroidism because the [tT_4] decreases more than the detectable decrease in [TSH].

II. Reported results
   A. Reported ratios have been calculated directly from the numerical value of reported concentrations, e.g., a [tT_4] of 10 nmol/L, a [fT_4] of 5 pmol/L, and a [TSH] of 0.5 ng/mL would result in a tT_4:TSH ratio of 20 nmol/μg (mmol/g) and a fT_4:TSH ratio of 10 pmol/μg (μmol/g).
   B. tT_4:TSH ratios (as nmol:μg) in one study showed that hypothyroid dogs usually had lower ratios (values of 1–66, with 10 of 11 ratios < 30) than healthy euthyroid dogs (values of 36–173) and sick euthyroid dogs (values of 45–2114; a ratio > 4000 was an obvious outlier).[182]
   C. tT_4:TSH and fT_4:TSH ratios in a 1999 study[183]
      1. tT_4:TSH ratio: Using a decision threshold of 17.3 (nmol/μg), the tT_4:TSH ratio had a diagnostic sensitivity for hypothyroidism of 86.7 % and a diagnostic specificity of 92.2 %.
      2. fT_4:TSH ratio: Using a decision threshold of 7.5 (pmol/μg), the fT_4:TSH ratio had a diagnostic sensitivity for hypothyroidism of 80.0 % and a diagnostic specificity of 97.4 %.
      3. Ratios and diagnostic performance vary with specific assays, laboratories, and decision threshold values.
   D. The ratios have not been widely used, and their diagnostic value compared to other assessments is not firmly established. Inaccurate [TSH] at very low concentrations (the assay's detection limit is near concentrations found in euthyroid dogs) could result in inaccurate and perhaps misleading ratios.

INTERPRETATION OF THYROID HORMONE CONCENTRATIONS AND PROFILES

I. Dogs
   A. Major patterns of thyroid profile results in dogs (Table 17.5)
   B. [tT_4] can be used to help monitor levothyroxine therapy at least 2 wk after beginning therapy or changing the dose. For dogs receiving appropriate thyroxine supplementation, [tT_4] is expected to be high-normal to increased 4–6 h after levothyroxine administration, and [TSH] should be WRI.

Table 17.5. Interpretation of selected thyroid profile results in dogs

$[tT_4]$	$[fT_4]_{ed}$	[TSH]	TgAA	Interpretation
WRI	—	—	—	Excludes hypothyroidism unless $[tT_4]$ is falsely increased by T_4AA
↓	↓	↑	Positive	Primary hypothyroidism caused by lymphocytic thyroiditis
↓	↓	↑	Negative	Primary hypothyroidism caused by thyroid atrophy or possibly end-stage immune thyroiditis
↓	↓	WRI[a]	Negative	Hypothyroidism, either primary with unexplained [TSH][b] or, rarely, secondary (pituitary gland dysfunction) NTI (sick euthyroidism) Hypothyroxinemia caused by effects of certain drugs[c] Breed variation (e.g., sighthounds) or geriatric dog
↓	WRI–↑[d]	WRI–↑	Negative	NTI (sick euthyroidism)
WRI–↑	↓	↑	Positive	Primary hypothyroidism (lymphocytic thyroiditis) with T_4AA increasing the $[tT_4]$
WRI	WRI	WRI	Positive	Thyroiditis (rarely neoplasia) without thyroid dysfunction, or false-positive TgAA (e.g., nonspecific binding)
WRI	WRI	↑	Positive	Thyroiditis with compensatory increase in TSH production
WRI	WRI	↑	Negative	Potentially responding after withdrawal of sulfonamides or levothyroxine; recovery from NTI that caused hypothyroxinemia

[a] The canine TSH assay lacks an adequate analytical range to measure decreased [TSH] accurately.
[b] Potential reasons for [TSH] to be WRI in a minority of dogs with primary hypothyroidism include concurrent NTI or drugs that suppress TSH release and perhaps variations in TSH molecules that reduce their detection.
[c] Glucocorticoids, phenobarbital, or clomipramine
[d] $[fT_4]_{ed}$ varies and may be decreased with severe illness. Results depend on where illnesses or drugs interfere with thyroid hormone production (see the text).
Note: Any $[tT_4]$ or $[fT_4]_{ed}$ values near respective reference limits (borderline results) should be interpreted cautiously.

C. To reassess a diagnosis of hypothyroidism via hormone assays in a dog receiving levothyroxine supplementation, levothyroxine should be discontinued for at least 1 wk prior to testing if on 26 μg/kg q24h for up to 16 wk, and for as long as 4 wk if receiving higher doses or for a longer duration. This will remove the influence of treatments on thyroid profile results.[26]

II. Major patterns of $[tT_4]$ and $[fT_4]$ in cats (Table 17.6)

Table 17.6. Interpretation of $[tT_4]$ and $[fT_4]_{ed}$ in cats

Clinical history and signs suggest:	$[tT_4]$	$[fT_4]_{ed}$	Interpretation
Hyperthyroidism	↑	—	Strong evidence of hyperthyroidism (a concurrently ↑ $[fT_4]$ provides additional evidence but is typically not needed)
	↓–WRI	WRI–↑	May be hyperthyroidism and/or NTI; T_3 suppression test recommended
Euthyroidism	WRI	WRI	No evidence of thyroid dysfunction
	↑	—	Could be early hyperthyroidism, individual biological variation, or inappropriate RI; a confirmatory ↑ $[fT_4]$ or a positive T_3 suppression test is needed before diagnosing hyperthyroidism
	↓	—	Probably NTI altering $[tT_4]$
	↓–WRI	↑	Probably NTI altering $[tT_4]$ or $[fT_4]$
	↓	WRI	Probably NTI altering $[tT_4]$
Hypothyroidism	↓	WRI–↑	NTI causing sick euthyroidism
	↓	↓	Probable primary hypothyroidism; could be secondary hypothyroidism or changes due to NTI or drug therapy

Note: Any $[tT_4]$ or $[fT_4]_{ed}$ values near respective reference limits (borderline results) should be interpreted cautiously.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 18

ADRENAL GLAND

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Table 18.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
17OHP	17α-hydroxyprogesterone
ACTH	Adrenocorticotropic hormone (corticotropin)
CLIA	Chemiluminescent immunoassay
CRH	Corticotropin-releasing hormone
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAN	Functional adrenal neoplasia
HAC	Hyperadrenocorticism (adrenal hypercortisolism)
HDDST	High-dose dexamethasone suppression test
IM	Intramuscularly
IV	Intravenously
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LDDST	Low-dose dexamethasone suppression test
NAI	Nonadrenal illness
o,p′DDD	1,1-Dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane
PDH	Pituitary-dependent hyperadrenocorticism
PPID	Pituitary pars intermedia dysfunction
RBC	Red blood cell (erythrocyte)
RI	Reference interval
RIA	Radioimmunoassay
SI	Système international d'unités (International System of Units)
UCCR	Urinary cortisol-to-creatinine ratio
WBC	White blood cell (leukocyte)
WRI	Within reference interval

PHYSIOLOGIC PROCESSES

I. Definitions
   A. *Steroids* are nonpolar molecules with a skeleton of four interconnected carbon rings that reflect their synthesis from cholesterol. They comprise glucocorticoids, mineralocorticoids, sex hormones, and their synthetic analogs.
   B. *Corticosteroids* are steroids produced by the adrenal cortices (also their synthetic analogs). They comprise glucocorticoids, mineralocorticoids, and some sex hormones; most sex hormones are produced in gonads.
   C. *Glucocorticoids* are corticosteroids synthesized in the adrenal zona fasciculata (also their synthetic analogs) that bind to glucocorticoid receptors and help regulate glucose metabolism. Cortisol is the major glucocorticoid in domestic mammals.
   D. *Mineralocorticoids* are corticosteroids synthesized in the adrenal zona glomerulosa (also their synthetic analogs) that help regulate Na^+, K^+, and H_2O balance. Aldosterone is the major mineralocorticoid.

II. Regulation of cortisol and aldosterone secretion (Figure 18.1)

A. CRH from the hypothalamus stimulates the production and release of pituitary ACTH and other hormones. Low [cortisol] promotes secretion of CRH and ACTH. High [cortisol] inhibits secretion of CRH and ACTH. Dopamine inhibits the secretion of ACTH; decreased dopamine secretion promotes synthesis and secretion of ACTH.[1] Vasopressin (ADH) augments the stimulatory effects of CRH on ACTH secretion; catecholamines, angiotensins, and serotonin also enhance CRH effects.[1]

B. Secretory pulses (~10–20/d) of ACTH stimulate the pulsatile production and release of cortisol, aldosterone, and other steroid compounds from the adrenal glands.[4,5] The adrenal gland cortices produce most circulating cortisol. Cortisol is synthesized in mitochondria through a series of enzymatic reactions that begin with cholesterol. Most of the cholesterol enters the adrenocortical cells from plasma lipoproteins; some is synthesized from intracellular stores of cholesteryl esters.

C. In adult horses, peak secretion of cortisol occurs in the morning and is lowest in the afternoon or evening,[6,7] whereas dogs and cats lack a clear circadian rhythm.[4] For horses, the degree of daily variation in [cortisol] may need to be considered when interpreting diagnostic tests;

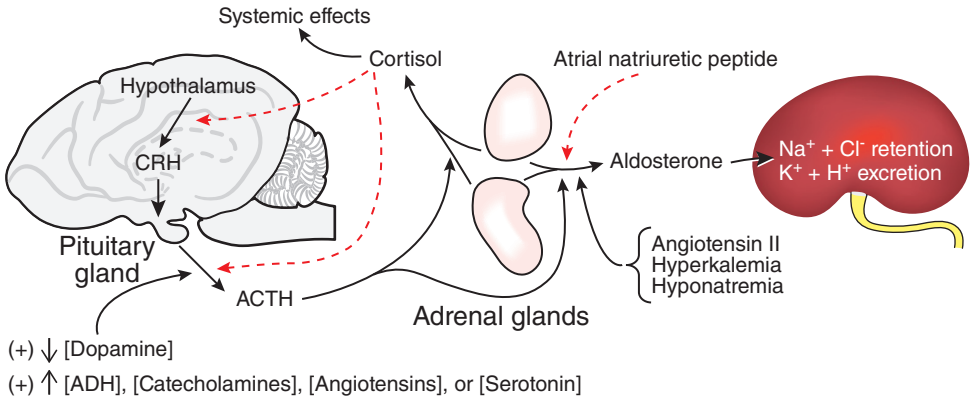


Figure 18.1. Regulation of cortisol and aldosterone secretion.

- In response to stress, CRH secreted from hypothalamic neurons enters hypothalamic capillaries and then the capillary bed of the anterior pituitary gland where it stimulates the production and release of ACTH from corticotrophs in the pars distalis. ACTH is also released from corticotrophs in the pars intermedia when [dopamine] decreases.[1]
- Within minutes, ACTH stimulates the production and release of cortisol and aldosterone from the adrenal gland cortices. In the negative-feedback system (*red dashed lines*), increasing the [cortisol] inhibits the secretion of CRH and ACTH. This CRH–ACTH–cortisol loop operates continuously in health, with pulses of ACTH followed shortly by increases in [cortisol] and then by decreases in [ACTH] and [cortisol].
- ACTH secretion is promoted by increased [ADH], [catecholamines], [angiotensins], or [serotonin].
- Increased [angiotensin II], hyperkalemia, hyponatremia, and increased [ACTH] promote the release of aldosterone from adrenal gland cortices. Atrial natriuretic peptide inhibits aldosterone release. The secreted aldosterone stimulates the renal retention of Na^+ and Cl^- and excretion of K^+ and H^+.
- Not shown: During inflammation, certain inflammatory cytokines can stimulate CRH, and it is likely that they can stimulate cortisol production directly (without ACTH).[2] Rarely, in disease, adrenal glands themselves can produce ACTH or express excessive or abnormal functional receptors for certain hormones (e.g., glucose-dependent insulinotropic peptide, ADH, luteinizing hormone) that stimulate cortisol production.[3]

differences might be as great as fourfold in an individual. Similar variation can occur between peaks and troughs of pulsatile cortisol concentrations, with the [cortisol] of pulsatile peaks commonly being 2–3 times the [cortisol] of troughs (data assessed for horses and dogs). This variation should be considered when interpreting cortisol results.

D. Adult male dogs have a greater mean plasma [cortisol] than adult female dogs;[8] RIs from some laboratories reflect this.

E. Most cortisol released from the adrenal glands becomes bound to plasma proteins, largely to corticosteroid-binding globulin (transcortin) and less to albumin, both of which are produced by hepatocytes. In healthy dogs and horses, the corticosteroid-binding globulin capacity for cortisol is about 2–5 times the plasma [cortisol].[9] If the binding capacity of cortisol-binding protein becomes saturated, more cortisol binds to albumin. Only ~10–15 % of plasma cortisol is free to enter cells and produce effects. This free cortisol also passes freely through the glomerular filtration barrier, but the majority is resorbed by distal renal tubular cells.[10] The plasma half-life of cortisol is about 1.5 h in dogs and horses,[11,12] and less in cats.

F. After cortisol enters cells, it binds to receptor proteins in cell nuclei. Cortisol-receptor complexes initiate synthesis of hormone and cytokine receptors and other proteins involved in gluconeogenesis, protein catabolism, lipolysis, immune responses, and H_2O balance.

G. The liver is an important site of cortisol inactivation and conjugation of metabolites to more-water-soluble forms that are excreted in urine. Conjugated cortisol metabolites are not resorbed from renal filtrate. Kidneys also inactivate cortisol.

III. Catecholamines

A. These organic amine neurotransmitters and hormones are synthesized in chromaffin cells of the adrenal medulla, gastrointestinal tract, and paraganglia areas. They comprise epinephrine (adrenaline), norepinephrine (noradrenaline), and dopamine. Dopamine is synthesized from the amino acid tyrosine. Norepinephrine is synthesized from dopamine, and methylation of norepinephrine yields epinephrine. The release of adrenal catecholamines is part of the "fight-or-flight response" of the sympathetic nervous system.

B. Spontaneous leaking of epinephrine and norepinephrine from storage granules (vesicles) into chromaffin cell cytoplasm leads to O-methylation (oxygen methylation) of the hormones to form metanephrine (metadrenaline) and normetanephrine (normetadrenaline), respectively.[13] Together, these are commonly referred to as metanephrines or O-methylated catecholamine metabolites. Most of the metanephrine and normetanephrine in blood was formed within chromaffin cells of the adrenal medulla. A major metabolite of dopamine is 3-methoxytyramine.

C. Plasma metanephrine and normetanephrine are rapidly cleared from plasma and metabolized, largely to sulfate-conjugated forms which return to plasma where they predominate over the unconjugated forms, which are called *free metanephrine* and *free normetanephrine*. Some free and all sulfated molecules of metanephrine and normetanephrine are renally excreted. In urine, over 95 % of the metanephrine and normetanephrine is conjugated. Vanillylmandelic acid is a metabolic end product of catecholamines that forms in the liver, mostly from catecholamine metabolites that entered blood after forming in neural tissue (neurons). Vanillylmandelic acid is excreted in urine.

ANALYTICAL CONCEPTS

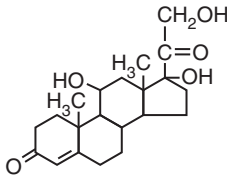
I. Unit conversion[14]

A. Cortisol: µg/dL × 10 = ng/mL and µg/dL × 27.6 = nmol/L (SI unit)

B. Aldosterone: ng/dL × 10 = pg/mL and ng/dL × 27.74 = pmol/L (SI unit)
C. ACTH: ng/L = pg/mL and pg/mL × 0.2202 = pmol/L (SI unit)
D. Epinephrine: pg/dL × 5.454 = pmol/L (SI unit)
E. Norepinephrine: pg/mL × 5.914 = pmol/L (SI unit)
F. Metanephrine: pg/mL × 5.07 = pmol/L (SI unit) ÷ 1000 = nmol/L
G. Normetanephrine: pg/mL × 5.46 = pmol/L (SI unit) ÷ 1000 = nmol/L

II. Sample

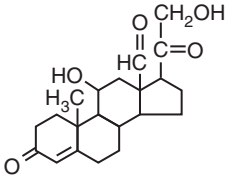
A. Cortisol (hydrocortisone)



Cortisol

1. Routine assays measure the total [cortisol], a minority (~10–15 %) being free (biologically active) and a majority (~85–90 %) being bound to protein. Concentrations of free cortisol can be measured after equilibrium dialysis. In urine, the measured cortisol is free unless there is proteinuria.
2. Serum or EDTA-plasma may be used. However, EDTA interferes when using a CLIA in which alkaline phosphatase generates luminescence (e.g., Immulite 1000 and 2000 analyzers), apparently by chelating the cations needed for the enzyme to function. Increased sample [EDTA] decreases the signal, and because the assay is competitive, this equates to a falsely increased [cortisol]. In a study of 40 samples from healthy dogs, there was a substantial proportional bias between serum and EDTA-plasma samples that was accentuated by underfilling the EDTA tubes (50 % of intended capacity).[15] The apparent [cortisol] was high enough (> 50 nmol/L) to support exclusion of hypoadrenocorticism in 17 dogs when testing serum, in 33 dogs when testing EDTA-plasma, and in all 40 dogs when testing EDTA-plasma from underfilled tubes. Samples from ill dogs were not assessed.
3. Stability of [cortisol] in EDTA-plasma is better than in sera, and better in cold samples (–20 °C best and 4 °C acceptable) than in warm ones (25 °C or 37 °C). The [cortisol] in hypercortisolemic serum samples had significantly decreased by day 5 of storage at 4 °C or higher (more so at higher temperatures), whereas there was no significant change for plasma samples stored at or below 25 °C for 5 d.[16] Samples (especially sera) should be shipped with ice packs.
4. Samples for urine [cortisol], including samples for a UCCR, should be kept at room or refrigeration temperature and shipped overnight with ice packs for testing within 3 d of collection.
5. It is occasionally written that serum or plasma should be collected from blood quickly because of the uptake of cortisol by RBCs. RBCs do bind cortisol that is added to blood,[17] but cortisol should already be distributed to plasma and RBCs in patient blood samples. There was no difference between measured [cortisol] in plasma samples removed from cells either 10 min or 40 h after blood collection.[18]

B. Aldosterone



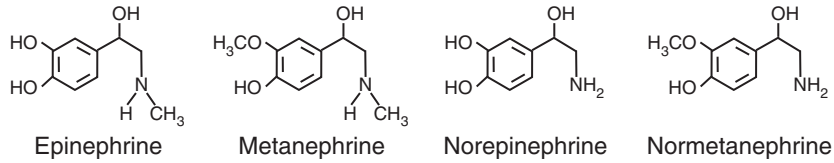
Aldosterone

1. EDTA-plasma, serum, and heparinized plasma have been used, but human studies suggest that different RIs may be appropriate for different sample types.[19] Follow your laboratory's guidelines.
2. In human studies, plasma and serum [aldosterone] was adequately stable for at least 5 d at 4 °C and for at least 48 h in

whole blood stored at room temperature.[19] Plasma or serum may be stored for at least 2 mo at −20 °C.

C. ACTH (corticotropin)
   1. EDTA-plasma is preferred. EDTA tubes should be filled to their intended capacity when using a CLIA in which alkaline phosphatase generates luminescence (e.g., Immulite 1000 and 2000 analyzers); the direct assay yields false decreases with underfilled tubes and therefore excess EDTA (see Cortisol Section A.2 above).[15] Plasma should be removed from the RBCs immediately, placed in a plastic tube, and chilled. The sample should not have prolonged contact with glass because ACTH adheres to glass.
   2. ACTH is very labile, so the plasma requires special handling.[20] If not analyzed the day of collection, then the sample should be frozen and shipped to the reference laboratory in a dry-ice shipment. Addition of a protease inhibitor such as aprotinin can prevent the ACTH degradation that occurs in refrigerated plasma; however, results of a CLIA differed with the presence or absence of aprotinin, with lower results in samples to which aprotinin was added.[21] Equine [ACTH] measured by a RIA was stable in EDTA-plasma (without aprotinin) for 3 h at 19 °C ,[22] and [ACTH] measured by a CLIA was reportedly stable in plasma collected from EDTA-blood stored for as long as 36 h at 7 °C prior to centrifugation.[23] It is best to harvest plasma promptly, refrigerate it, and test within 36 h, or freeze the sample if testing will be more delayed.

D. Catecholamines (epinephrine, norepinephrine) and their metabolites (metanephrine, normetanephrine)



   1. Stress, excitement, and activity before and during collection of blood or urine should be minimized because free metanephrine and free normetanephrine are rapidly produced and cleared from circulation, thus affecting plasma and urine measurements.[24] The patient should be free of drugs that may affect catecholamine production or metabolism (e.g., norepinephrine and serotonin uptake inhibitors, α- or β-adrenergic antagonists, dopamine or dopamine agonists).
   2. Blood samples are collected into chilled heparin or EDTA tubes which should be refrigerated for no more than 3–6 h prior to centrifugation at 4 °C. Plasma may be refrigerated for up to 3 d with only mild changes in results, but freezing (preferably at −80 °C) is required if analysis will be delayed longer, and it is recommended if testing will be delayed at all.
   3. Urine samples for catecholamine metabolites do not require acidification, but catecholamines degrade in alkaline urine, so if catecholamine concentrations are to be measured, prompt sample acidification is indicated. Free metanephrines assessed by LC-MS/MS (recommended) were reportedly stable without acidification in canine urine stored at 4 °C or room temperature for 2 d[25] and in feline urine stored at 4 °C for 24 h.[26] Samples should be collected and processed as recommended by a reference laboratory.

4. In human medicine, the renal excretion of these compounds is quantified via a 24-h urine collection. Because this is typically not practical in veterinary medicine, an aliquot of urine is used for analysis of the urine [creatinine] so that an analyte-to-creatinine ratio can be calculated (Eq. 18.1). A separate urine sample may be needed if urine is acidified. When interpreting patient results with published data, it is necessary to recognize how the ratio is expressed, i.e., as a unitless ratio or with units of nmol/mmol, μg/mg, or μg/g. Some published results do not state if calculated ratios are with units or unitless.

$$(\text{Metanephrine:Creatinine})_u \text{ ratio} = \frac{\text{urine [metanephrine]}}{\text{urine [creatinine]}}$$

with [metanephrine] in nmol/L and [creatinine] in mmol/L; u, urine

Example: urine [metanephrine] = 1000 nmol/L; urine [creatinine] = 10 mmol/L

$$(\text{Metanephrine:Creatinine})_u \text{ ratio} = \frac{1000 \text{ nmol/L}}{10 \text{ mmol/L}} = 100 \text{ nmoL/mmoL (with units)}$$

$$= \frac{1000 \text{ nmol/L}}{10 \times 10^6 \text{ nmol/L}} = 100 \times 10^{-6} \text{ (unitless ratio)}$$

(**18.1**)

III. Principles and assay procedures

A. Cortisol

1. For several decades, RIAs were the gold standard and common clinical assays, but they have largely been replaced in clinical laboratories by enzyme immunoassays (ELISAs, CLIAs, and fluorometric assays). A SNAP ELISA was found to have enough disagreement with other methods to warrant retesting of equivocal samples at a reference laboratory.[27] One dry-chemistry cartridge immunoassay for canine cortisol uses surface plasmon-enhanced fluorescence, but no published independent validation study was found. High-performance liquid chromatography (HPLC) and LC-MS/MS may also be used, allowing greater analytical specificity and concurrent detection of multiple molecules. Whatever the assay, RIs and decision thresholds should be developed or verified for each laboratory and analyzer.
2. Anti-cortisol antibodies used in immunoassays generally cross-react with some other steroid molecules, but plasma concentrations of endogenous cross-reactive molecules are relatively low. Unless the cross-reactivity of an assay is known, a [cortisol] should be interpreted with caution if the animal has received corticosteroid therapy. The following cross-reactivity percentages are for a common CLIA (manufacturer's data): prednisolone (62 %), methylprednisolone (22 %), prednisone (6.1 %), and dexamethasone (< 0.1 %).[28] The manufacturer's data for a new (in 2021) anti-cortisol antibody used with the same analyzer indicated cross-reactivity percentages of 23.8 % for prednisolone and 1.0 % for methylprednisolone.[29] Poor cross-reactivity with dexamethasone is important for dexamethasone suppression tests.
3. Urine cortisol: Assays designed to measure plasma [cortisol] are also used to measure urine [cortisol] for a UCCR. However, urine contains cortisol metabolites and other steroidal compounds that may react with the anti-cortisol antibodies. The degree of cross-reactivity varies among assays (antibodies),[30] but immunoassays for urine

[cortisol] generally overestimate the true [cortisol], sometimes substantially.[31] Solvent extraction of polar metabolites and chromatography have been applied to urine samples to increase analytical specificity of immunoassay measurements for [cortisol], but they are usually not used. LC-MS/MS would be more analytically specific. Without knowing what other steroidal molecules are present and their degrees of cross-reactivity, it is impossible to know the degree of inaccuracy of a measured urine [cortisol].

a. In a study using four different immunoassays, significant cross-reactivity was found with cortisone, 11-deoxycortisone, 5β-dihydrocortisol, and 5α-tetrahydrocortisol; cross-reactions were less with other steroidal compounds.[32]
b. In a comparison of five immunoassays with results of a gas chromatography-mass spectrometry method, each immunoassay had a positive bias for measuring cortisol in canine urine.[31] The authors stated the positive biases were because of cross-reactions with other steroidal molecules, but they were not identified.

B. ACTH
1. Immunoradiometric assays, CLIAs, and immunofluorescence immunoassays have been used to measure [ACTH] in dogs, cats, and horses. Different assays can yield different results, so interpretation should be guided by laboratory-specific RIs and decision thresholds.[21,33]
a. Antibodies used in immunoassays designed for measuring human ACTH tend to cross-react with canine, feline, and equine ACTH.
b. However, antibody reactivity varies. Some antibodies are specific for intact equine ACTH, but others lack analytical specificity and detect other peptides released by the equine pars intermedia, e.g., corticotropin-like intermediate lobe peptide (CLIP).[34,35]
2. Instability of ACTH is the major concern with ACTH measurement. Samples must be processed appropriately to obtain valid results.

C. Aldosterone
1. Aldosterone RIAs are not as common as cortisol assays. Concentrations of aldosterone are relatively minute, requiring assays with low limits of quantitation (pg/mL) and high analytical sensitivity.
2. Physiologic and dietary factors (hydration state, salt intake) that influence aldosterone production need to be considered when establishing RIs for [aldosterone].

D. Catecholamines (epinephrine, norepinephrine) and their metabolites (metanephrine, normetanephrine)
1. Historically, catecholamine production was assessed by measuring urine concentrations of the catecholamines themselves, but they are unstable. Later, the urine concentration of vanillylmandelic acid was measured because of its greater stability and greater concentration in urine, but much of it is produced from catecholamines that are not derived from the adrenal gland, making it less specific for adrenal disorders. Assays were then developed to measure urine concentrations of total metanephrine and total normetanephrine concentrations; these total concentrations comprise the free and conjugated forms, the latter requiring acid hydrolysis or enzymatic deconjugation for measurement (other terms for total metanephrine and total normetanephrine are deconjugated metanephrine and deconjugated normetanephrine). More recently, methods were developed that allow quantitation of the low concentrations of free metanephrine and normetanephrine in plasma and urine.

2. Concentrations of catecholamines, metanephrine, and normetanephrine have been measured with immunoassays, gas chromatography, high-pressure liquid chromatography with electrochemical detection, and LC-MS/MS. LC-MS/MS is currently considered the method of choice, where available, and has been used to measure concentrations of free metanephrine and free normetanephrine in canine and feline plasma and urine.[26,36,37] Interpretation of results requires valid RIs or decision thresholds for each assay.

CORTISOL CONCENTRATION

I. Disorders and conditions that cause hypercortisolemia (Table 18.2)
   A. Spontaneous conditions of increased cortisol production: Unstimulated (basal) plasma [cortisol] by itself is of limited value because many patients with HAC do not maintain an increased [cortisol], and when increases are present, they are frequently mild increases that can be found in physiologic states. However, there is sufficient cortisol production to cause clinical disorders either because of episodic exaggerated secretions or because of a mild but continuously increased production of cortisol. A [cortisol] is best interpreted in either dexamethasone suppression tests or ACTH stimulation tests (see later sections). Historical, physical, and preliminary laboratory data may clearly indicate that an animal has HAC but may not clearly differentiate the causes of the pathologic state. Adrenal suppression and stimulation tests (covered later in this chapter) can be helpful for this, and diagnostic imaging methods (radiography, ultrasonography, computed axial tomography, or magnetic resonance imaging) can locate the pituitary or adrenal neoplasms but cannot determine whether the abnormal tissue is producing excessive amounts of hormones. Published guidelines are available to aid the diagnosis and treatment of canine and feline hypercortisolism.[38]

Table 18.2. Disorders and conditions that cause hypercortisolemia

Spontaneous conditions of increased cortisol production
ACTH-dependent HAC
PDH*
Ectopic production of ACTH by a neoplasm
Bilateral nodular adrenal cortical disease (food-dependent HAC, other)
ACTH-independent HAC
FAN:* adenoma, adenocarcinoma
"Occult hyperadrenocorticism"
Ovarian steroid cell tumor
Stress-induced hypercortisolemia*
Iatrogenic conditions of increased cortisol production
Exogenous ACTH administration

* A relatively common disorder, condition, or mechanism
Note: Pharmacologic doses of some steroid compounds (not dexamethasone) may cross-react in the cortisol assay and falsely elevate [cortisol].

1. ACTH-dependent HAC
   a. PDH (Figure 18.2B): This form of HAC is commonly referred to as Cushing's disease, the name originally given to the human pituitary disorder described by Dr. Harvey Cushing.
      (1) In PDH, the neoplastic pituitary cells release excess ACTH, which causes bilateral adrenocortical hyperplasia and excessive cortisol production. The ACTH release may or may not be inhibited by exogenous glucocorticoids. Individual neoplasms can be inhibited by some steroids but not others.
      (2) In dogs, most cases of clinical HAC (> 90 % in some reports) are caused by increased release of ACTH from pituitary corticotroph adenomas. A small percentage are caused by pituitary adenocarcinomas or pituitary hyperplasia.[39] Rarely, ACTH-producing pituitary masses other than corticotroph adenomas may cause HAC.
      (3) In cats, about 75–85 % of clinical HAC cases are pituitary dependent and caused by increased release of ACTH from pituitary adenomas or, rarely, pituitary adenocarcinomas or multiple pituitary neoplasms.[40–42]
      (4) HAC is rare in horses.[43] However, older horses commonly develop PPID caused by an adenoma or hyperplasia of the pars intermedia. Although this condition was historically considered HAC (Cushing's disease), hypercortisolemia is generally not present.[44] There is evidence that PPID is secondary to hypothalamic degeneration that decreases dopamine production. Dopamine from dopaminergic neurons inhibits the melanotropes of the pars intermedia. Without this inhibition, the melanotropes produce excess pro-opiomelanocortin, which is cleaved into β-melanocyte–stimulating hormone (β-MSH), corticotropin-like intermediate lobe peptide, β-endorphin–related peptide (βEND peptide), and ACTH. The melanotropes become hyperplastic or neoplastic.
   b. Ectopic production of ACTH by a neoplasm: Neoplastic tissue other than pituitary neoplasms can produce ACTH that causes bilateral adrenocortical hyperplasia and clinical HAC.[45,46] These disorders are rarely recognized in domestic mammals but have been found in people for many years.
   c. Bilateral nodular adrenal cortical disease: This is a heterogeneous group of rare but increasingly recognized genetic disorders in people with germline mutations that probably promote later somatic adrenal mutations leading to subclinical or clinical hypercortisolemia.[3] Increased cortisol secretion is associated with abnormal intra-adrenal ACTH production and excessive or ectopic adrenocortical cell expression of functional receptors for various hormones, e.g., glucose-dependent insulinotropic polypeptide (GIP, also called gastric inhibitory polypeptide), ADH, luteinizing hormone, human chorionic gonadotropin hormone, leptin, serotonin, angiotensin, and catecholamines. Stimulation of these aberrant receptors coupled with stimulation by locally produced ACTH causes excessive cortisol secretion. Hypercortisolemia, unless mild, suppresses pituitary ACTH production, giving the impression of an ACTH-independent adrenal proliferation. However, local (paracrine) ACTH appears to be involved, so the disorder is no longer considered ACTH-independent. Rare reports support the occurrence of this condition in dogs.

(1) Food-dependent HAC was diagnosed in a 6-yr-old dog with (i) clinical signs of HAC, (ii) bilateral adrenomegaly with no pituitary abnormality based on imaging, (iii) meal-induced increases in plasma [cortisol] and UCCR, (iv) decreased [ACTH], (v) prevention of the meal-induced increases in plasma [cortisol] by administration of octreotide, and (vi) a positive response to treatment with trilostane.[47] Octreotide is a somatostatin analog that inhibits secretion of many peptides, including GIP. In this condition, food ingestion stimulates release of GIP, which then binds to aberrant GIP receptors on adrenocortical cells and stimulates cortisol secretion. This dog's adrenal glands were not visualized macroscopically or assessed histologically. The genetic causes of most human cases of food-dependent HAC have been identified.[48]

(2) Bilateral nodular adrenal cortical disease unrelated to meals was suspected in a hypercortisolemic dog with decreased [ACTH], no pituitary lesion based on imaging and necropsy, and with immunohistochemical evidence of ACTH production by adrenocortical cells.[49] The dog also had a pheochromocytoma that did not appear to express ACTH based on immunohistochemistry.

(3) In a study examining the potential role of adrenal luteinizing hormone receptors in HAC, a subset of spayed dogs diagnosed with HAC had exaggerated increases in [cortisol] after administration of human chorionic gonadotropin (which binds luteinizing hormone receptors), and these dogs also had (i) lower cortisol responses to ACTH, (ii) failure of cortisol suppression by dexamethasone, and (iii) endogenous ACTH concentrations that were not increased.[50] The affected dogs had been spayed when 5–9 yr old, mostly later in life than the other subgroup of dogs with HAC. Also, ACTH stimulation testing of 16 apparently healthy dogs of similar ages showed an increase in baseline and post-ACTH [cortisol] 60 d after ovariohysterectomy. An increase in plasma [luteinizing hormone] after ovariectomy might cause or contribute to HAC in some dogs.

2. ACTH-independent HAC
   a. FAN, also referred to as a functional adrenocortical tumor (FAT) or adrenal-dependent HAC (ADH, avoided here because it is also the abbreviation for antidiuretic hormone) (Figure 18.2C)
      (1) With FAN, cells of an adrenocortical adenoma or adrenocortical carcinoma produce excess cortisol. The neoplastic cells may or may not be responsive to exogenous ACTH. A high [cortisol] is expected to inhibit ACTH release, thereby promoting atrophy of the nonneoplastic adrenal gland.
      (2) In dogs, about 15–20 % of clinical HAC cases are due to adrenal adenomas or adenocarcinomas.[51] Clinical signs of HAC may occur even when adrenocortical neoplasms produce increased amounts of noncortisol steroid hormones without apparent increases in cortisol (see Concentrations of Adrenal Sex and Intermediate Hormones).
      (3) In cats, about 15–25 % of clinical HAC cases are due to adrenal adenomas or adenocarcinomas.[40,41] Clinical signs of HAC may occur even when adrenocortical neoplasms produce increased amounts of noncortisol steroid hormones without apparent increases in cortisol (see Concentrations of Adrenal Sex and Intermediate Hormones).

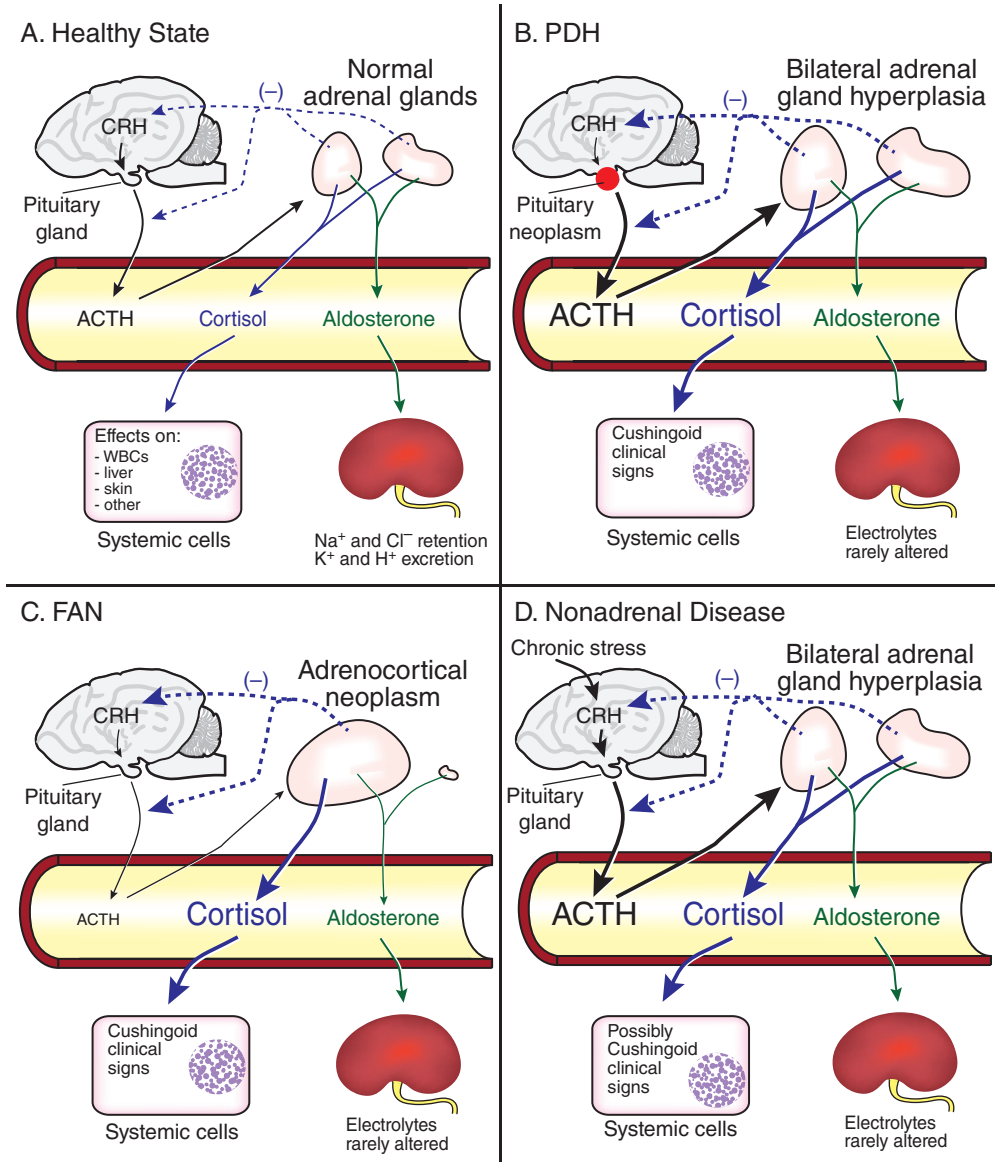


Figure 18.2. Common hyperadrenocorticism disorders.

A. Healthy state: CRH released from the hypothalamus stimulates the release of ACTH from the pituitary gland. ACTH stimulates the release of cortisol by the adrenal cortices; cortisol inhibits the release of CRH and ACTH, and alters the metabolism of many systemic cells including WBCs, hepatocytes, and skin cells. To a lesser extent, ACTH stimulates the release of aldosterone by the adrenal cortices; aldosterone promotes the retention of Na^+ and Cl^- and the excretion of K^+ and H^+ by renal tubules.

B. PDH: Excessive ACTH secretion by a pituitary neoplasm creates bilateral adrenal gland hyperplasia, release of cortisol, and potentially hypercortisolemia. Cushingoid clinical signs are created by the effects of cortisol on systemic cells. There may be increased aldosterone release, but electrolyte concentrations are typically not altered because of other factors regulating electrolytes.

b. "Occult HAC"
   (1) The canine disorder has been defined as "a syndrome in which a dog appears to have HAC based on history, physical examination, and clinical laboratory findings, but the LDDST, UCCR, and ACTH stimulation test fall into currently accepted reference ranges."[52] In other words, clinical signs of HAC are not occult, but the diagnosis is occult based on typical laboratory test results. These dogs may have an adrenocortical neoplasm that is secreting steroid hormones other than cortisol, but other biological variations or test limitations may mask the diagnosis of HAC.
   (2) Diagnosis of this pathologic state frequently involves measurement of several steroidal hormones (e.g., progesterone, 17OHP, estradiol).[52]

c. Ovarian steroid cell tumor in a dog: Ovarian neoplasms can produce steroids that cause clinical HAC. The dog in this report had clinical findings suggestive of HAC, lack of cortisol suppression with a LDDST, and a successfully treated hypoadrenal crisis after ovariohysterectomy.[53]

3. Stress-induced hypercortisolemia (Figure 18.2D)
   a. Illness, surgical or unintentional trauma, or environmental changes may stimulate the release of CRH, then ACTH, and thus stimulate the adrenal glands to produce more cortisol.
   b. If persistent, the stress response may lead to bilateral adrenocortical hyperplasia, but it should not cause the clinical manifestations of pathologic HAC. However, the animal's primary pathologic state may be creating clinical signs similar to those of HAC.
   c. With critical illness (e.g., sepsis and systemic inflammatory response syndrome), decreased hepatic and renal expression of cortisol-metabolizing enzymes appears to contribute to the commonly increased concentrations of free and total cortisol reported in people and dogs, and inflammatory cytokines may also stimulate cortisol production directly (not via ACTH).[2,54] However, the [cortisol] of some critically ill patients is decreased rather than increased; cortisol inactivation may contribute.[55]
   d. Although not described in veterinary medicine, hypercortisolemia related to NAI might be masked by decreases in protein-bound cortisol caused by decreased plasma concentrations of cortisol-binding globulin or albumin. Routine assays measure [total cortisol] rather than the biologically important [free cortisol], so free hypercortisolemia can be present when hypoproteinemia reduces the bound fraction enough that the [total cortisol] is not increased.[56]

Figure 18.2. *continued*

C. FAN: Excessive cortisol secretion by an adrenocortical neoplasm potentially causes hypercortisolemia. The excessive cortisol inhibits release of CRH and ACTH; subsequently reduced ACTH secretion leads to hypoplasia of the other adrenal gland. Cushingoid clinical signs are created by the effects of cortisol on systemic cells. Aldosterone secretion can continue by adrenal cortical cells that are not neoplastic.

D. NAI: Many disorders and conditions create chronic stress that causes increased hypothalamic release of CRH and subsequently release of ACTH. Excessive ACTH secretion creates bilateral adrenal gland hyperplasia, causes a release of cortisol, and potentially creates hypercortisolemia. Cushingoid clinical signs are not expected but may be present. Aldosterone release may be increased but electrolyte concentrations are typically not altered.

B. Iatrogenic conditions of increased cortisol production: Exogenous ACTH administration is expected to cause hypercortisolemia (see Suppression and Stimulation Tests for Assessing Adrenocortical Responses, Section I.D).

C. Because the anti-cortisol antibody in immunoassays may cross-react with other steroids, recent administration of steroids could result in a falsely increased measured concentration. For example, assays that list significant cross-reactivity with prednisolone may have false values if the animal was recently treated with prednisolone (e.g., Prednis-Tab), prednisone (e.g., Meticorten), or prednisolone sodium succinate (e.g., Solu-Delta-Cortef). Other potential causes of falsely increased [cortisol] include increased cortisol-binding globulin in samples tested with competitive immunoassays (cortisol-binding protein can compete excessively with reagent cortisol, so less is available to compete with patient cortisol).

D. Common routine laboratory findings with hypercortisolemia
   1. Leukogram: steroid leukogram (see Chapter 2)
   2. Chemistry profile: increased serum activity of alkaline phosphatase (ALP) in dogs, hyperglycemia, hypercholesterolemia, and lipemia
   3. Urinalysis: relatively unconcentrated, isosthenuric, or hyposthenuric urine, proteinuria, and glucosuria

II. Disorders and conditions that cause hypocortisolemia (Table 18.3)

A. Spontaneous conditions of decreased cortisol production: Unstimulated plasma [cortisol] may be used as a screening test for hypoadrenocorticism in dogs. More than 96 % of dogs with clinical findings suggestive of hypoadrenocorticism and unstimulated plasma [cortisol] > 55 nmol/L (2 μg/dL) do not have hypoadrenocorticism; more than 93 % with an unstimulated plasma [cortisol] > 22 nmol/L (0.8 μg/dL) do not have hypoadrenocorticism.[28] Similar data have been reported by others.[57,58] The lower the decision threshold is set, the less likely a subthreshold value will be from a dog without hypoadrenocorticism, but some dogs with NAI have comparably low values.[28,59] Therefore, an unstimulated plasma

Table 18.3. Disorders and conditions that cause hypocortisolemia

Spontaneous conditions of decreased cortisol production
Primary hypoadrenocorticism*
Secondary hypoadrenocorticism (ACTH deficiency)
Primary hypoadrenocorticism with selective cortisol deficiency
Critical illness
Iatrogenic conditions of decreased cortisol production
Iatrogenic hypercorticism[a]*
Iatrogenic hypoadrenocorticism[b]
Ketoconazole or trilostane treatment
Sudden withdrawal of glucocorticoid treatments

[a] The animal has clinical manifestations (e.g., polyuria or alopecia) of excess glucocorticoid hormonal activity because of prolonged treatment with glucocorticoid compounds.

[b] The animal has clinical manifestations of deficiencies in glucocorticoid and mineralocorticoid hormonal activity (e.g., weakness, azotemia, hyponatremia, hypochloremia, or hyperkalemia) caused by adrenocortical hypoplasia induced by o,p'DDD, trilostane, or sudden withdrawal of prolonged treatment with glucocorticoid compounds (e.g., prednisolone, prednisone, or megestrol acetate).

[cortisol] below any given decision threshold is not diagnostic for hypoadrenocorticism; an ACTH-stimulation test is considered the confirmatory test for canine hypoadrenocorticism. Guidelines are available to aid the diagnosis and treatment of canine hypercortisolism.[38]

1. Primary hypoadrenocorticism (Figure 18.3B): This form of hypoadrenocorticism is commonly referred to as Addison's disease, the name originally given to the human adrenal disorder described by Dr. Thomas Addison.
   a. Causes of spontaneous bilateral atrophy or destruction of adrenal glands are usually not known, but they may include immune-mediated disease, adrenalitis, and rarely bilateral metastatic neoplasms, adrenal hemorrhage, or adrenocortical necrosis.
   b. The disorder is recognized primarily in dogs but also occurs in cats[60,61] and occasionally in foals.[62] Genetic predispositions have been identified in dogs, and detection of lymphocytic adrenal infiltrates and specific autoantibodies in some dogs supports an immune-mediated pathogenesis.
   c. The majority of dogs with primary hypoadrenocorticism have serum electrolyte abnormalities supportive of hypoaldosteronism (increased [K^+], decreased [Na^+], or both), but these electrolyte changes are absent in others. Cases with these electrolyte disorders have often been referred to as *typical*; cases without these electrolyte abnormalities have often been referred to as *atypical*. These terms should not be interpreted to mean that aldosterone is affected only in "typical" hypoadrenocorticism; decreased aldosterone responses to exogenous ACTH stimulation have been documented in dogs with "atypical" hypoadrenocorticism, and aldosterone has not been evaluated in most hypoadrenal dogs lacking electrolyte abnormalities.[63]
2. Secondary hypoadrenocorticism (ACTH deficiency): ACTH deficiency could result from destruction of the pituitary gland by a variety of disease processes (vascular, inflammatory, neoplastic, traumatic, or developmental) including surgery, but these are much less common than primary hypoadrenocorticism.
3. Primary hypoadrenocorticism with selective cortisol deficiency: In this disorder, spontaneous hypocortisolemia occurs without concurrent hypoaldosteronemia, hyponatremia, or hyperkalemia.[64] This may occur if the zona glomerulosa (responsible for mineralocorticoid production) is relatively preserved despite atrophy of the other adrenocortical layers.[65]
   a. In most cases of hypoadrenocorticism without electrolyte changes, aldosterone concentrations were not reported, and cortisol deficiency may not have been selective despite lack of electrolyte changes.[66,67] Aldosterone reserve can be reduced without electrolyte changes,[63] and electrolyte changes may develop with disease progression.[67]
   b. Concurrent cortisol and thyroxine deficiencies were considered a polyglandular deficiency syndrome in a dog with histologic evidence of a relatively unaffected zona glomerulosa with loss of the other cortical zones; [aldosterone] was not reported.[68]
4. Critical illness: Cortisol secretion is typically increased with the stresses of illness, but it may appear decreased with a low [total cortisol] and an apparent diminished response to exogenous ACTH if the protein-bound fraction of plasma cortisol is decreased (see Suppression and Stimulation Tests for Assessing Adrenocortical Responses, Section I.D.6.d).[56] Secretion may be truly reduced if prolonged illness and increased cortisol secretion first suppressed production of ACTH and caused adrenocortical atrophy.[69]

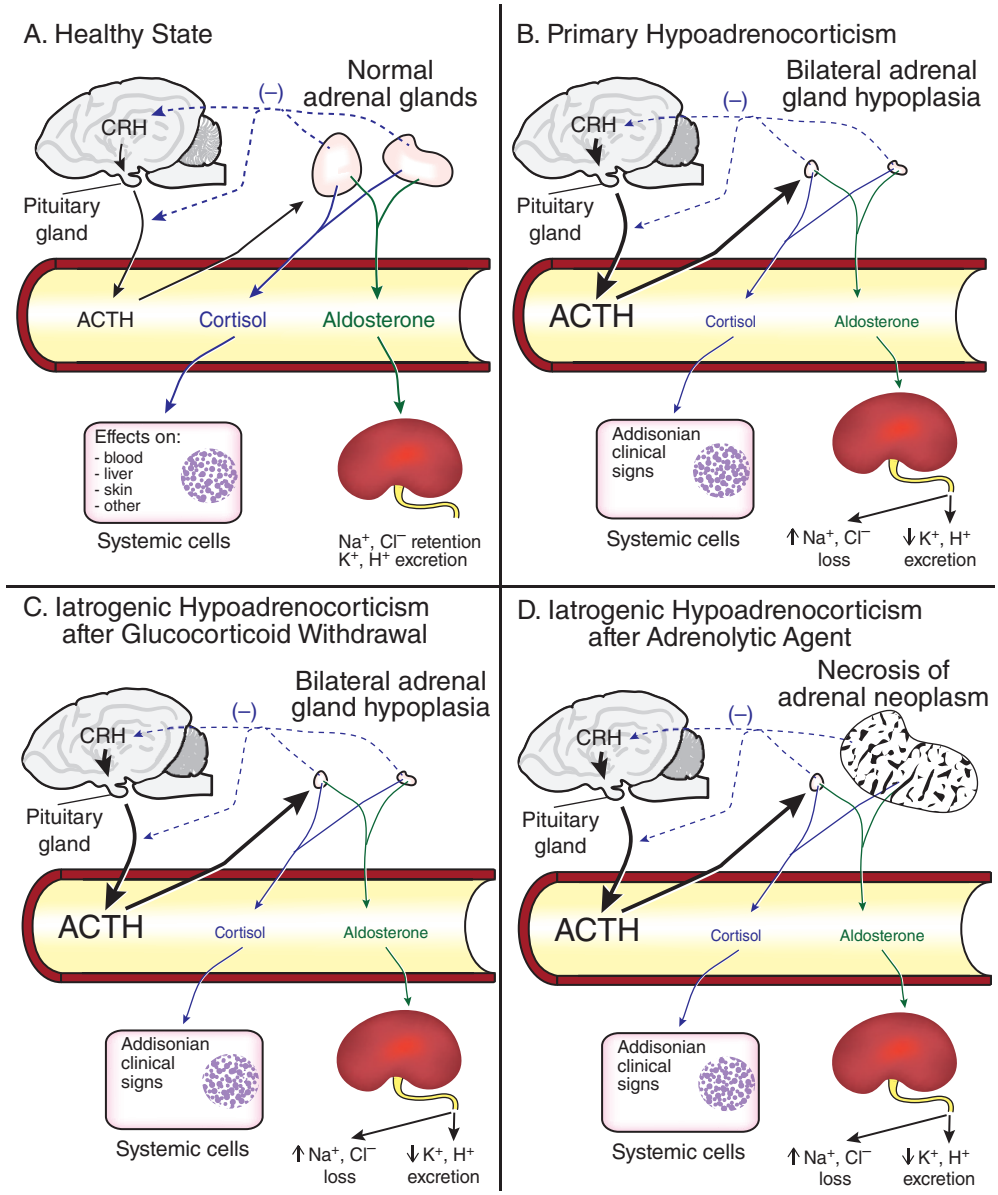


Figure 18.3. Common hypoadrenocorticism disorders.

A. See caption for Figure 18.2A.

B. Primary hypoadrenocorticism: Reduced aldosterone secretion by hypoplastic adrenal glands increases renal loss of Na^+ and Cl^- and decreased renal excretion of K^+ and H^+. Greatly reduced cortisol secretion leads to hypocortisolemia which contributes to a clinical Addisonian disorder (see Chapter 9). With less negative feedback, there is increased release of CRH and ACTH.

C. Iatrogenic hypoadrenocorticism after glucocorticoid withdrawal: Persistent treatment with a glucocorticoid (e.g., prednisolone, prednisone) reduces ACTH secretion and may therefore create bilateral adrenal gland hypoplasia. Subsequently, sudden withdrawal of glucocorticoid treatments may result in manifestations similar to primary hypoadrenocorticism (see above) despite increased ACTH production, because there is a lack of adrenal cortical mass to respond.

D. Iatrogenic hypoadrenocorticism after an adrenolytic agent: Administration of *o,p'*DDD (mitotane, Lysodren) to treat an adrenocortical neoplasm (shown) or bilateral adrenocortical hyperplasia (not shown) causes necrosis of adrenocortical cells. If too many functional cortical cells are destroyed, bilateral adrenal gland hypoplasia is created. Manifestations similar to primary hypoadrenocorticism may appear (see above).

B. Iatrogenic conditions of decreased cortisol production
   1. Iatrogenic hypercorticism
      a. Chronic administration of glucocorticoid drugs (e.g., prednisone, prednisolone, or topical clobetasol propionate) may produce clinical signs of hypercortisolism.
      b. The exogenous glucocorticoids cause negative feedback on the release of CRH and ACTH, and thus adrenal glands atrophy and produce less cortisol. Baseline [cortisol] typically is decreased (< 1.0 μg/dL), and the ACTH stimulation test response is diminished. Slow withdrawal of the steroid treatments enables the adrenal glands to recover.
   2. Iatrogenic hypoadrenocorticism (Figures 18.3C and D)
      a. Treatment of dogs with o,p′DDD (mitotane, Lysodren) for HAC (due to PDH or FAN) causes progressive necrosis of the adrenal cortex (zona fasciculata and zona reticularis). Excessive necrosis can decrease adrenal function and cause clinical hypoadrenocorticism with cortisol deficiency and sometimes mineralocorticoid deficiency.
      b. Treatment of dogs with trilostane can cause hypocortisolemia, and it rarely causes clinical iatrogenic hypoadrenocorticism.[70] Trilostane reduces [cortisol] by inhibiting 3β-hydroxysteroid dehydrogenase, an enzyme that catalyzes an intermediate reaction in steroid synthesis.[71] The mechanism by which it causes permanent iatrogenic hypoadrenocorticism is not known.
      c. Treatment of dogs or cats with ketoconazole can cause decreased [cortisol] because ketoconazole inhibits many steps in steroid biosynthesis.[72,73] Low- and high-dose ketoconazole treatments have been suspected to cause clinical iatrogenic hypoadrenocorticism in dogs.[74,75]
   3. Sudden withdrawal of glucocorticoid treatments (including megestrol acetate in cats) may cause clinical hypoadrenocorticism because of adrenal gland atrophy that developed while the animal was receiving glucocorticoids.
   4. A routine cortisol measurement yields a [total cortisol], the majority of which is protein bound. Hypoalbuminemia, a decreased concentration of cortisol-binding globulin, or both might falsely suggest a hypocortisolemic state when [free cortisol] is actually WRI or increased (see Suppression and Stimulation Tests for Assessing Adrenocortical Responses, Section I.D.6.d). This has been reported in people with critical illness (e.g., sepsis).[56]

C. Common routine laboratory findings with hypocortisolemia (except with iatrogenic hypercorticism caused by glucocorticoid administration)
   1. Leukogram: absence of steroid leukogram in stressed, ill patients (particularly lack of lymphopenia and lack of an eosinophil concentration at or near 0/μL, possibly with lymphocytosis or eosinophilia)
   2. Chemistry profile: hyponatremia, hypochloremia, hyperkalemia, hypercalcemia, hypoglycemia, and azotemia
   3. Urinalysis: inappropriately low urine specific gravity with azotemia

URINE CORTISOL (CORTICOID)-TO-CREATININE (UCC) RATIO

I. As described in Chapter 8, comparison of a urine analyte's concentration to urine [creatinine] provides a relative rate of urinary excretion of the analyte (such as cortisol) compared to creatinine. In theory, the UCCR should detect a hyperadrenal state better than a single unstimulated serum [cortisol] because the cortisol that accumulates in urine was produced over a longer period

and thus is not as susceptible to variations caused by pulsatile cortisol secretions. Pulsatile secretions can cause fluctuations in serum [cortisol]. However, increased urinary cortisol excretion could be either physiologic or pathologic.

A. Urine sample and analysis
   1. At least 1.0 mL (varies with laboratory) of supernatant from a urine sample should be submitted for analysis. Because stressful conditions can increase renal excretion of cortisol, it is preferred that the sample be collected as an early morning sample at home and a day or two after stressful states (e.g., a trip to a veterinary clinic!).[76] In a study of clinically healthy dogs, the median UCCR was 17 % greater in voided samples collected at a veterinary clinic after patient examination than in voided samples collected by the owner at home earlier in the day; the UCCR increased at least 10× for some dogs, and was greater than the reference limit for 7 of the 36 samples collected in-clinic but for none collected at home.[77]
   2. Anti-cortisol antibodies vary among immunoassays and may cross-react with chemically related compounds in urine (see Analytical Concepts, Section III.A.3). Consequently, the test is also referred to as the urine *corticoid to creatinine ratio*, corticoids including all steroids produced in the adrenal cortex (or their synthetic analogs), and in this context, also their metabolites. In the absence of pharmacologic interferences, the urine corticoid concentration measured by immunoassays generally reflects adrenal cortisol production, so immunoassays have diagnostic utility despite nonsepecificity.

B. The UCCR should be calculated by using molar concentrations of urine cortisol and creatinine (Eq. 18.2). A markedly different ratio would result if [cortisol] and [creatinine] were expressed as μg/dL and mg/dL, respectively. Instead of stating the true ratio value (e.g., 20×10^{-6}), it may be truncated to "20" in clinical jargon, or reported with other exponents, e.g., 2.0×10^{-5} rather than 20×10^{-6}.

C. Because of differences in assay methods and antibodies, valid RIs and medical decision thresholds determined for one assay and laboratory may not be applicable to others.[78]

$$\text{UCCR} = \frac{\text{urine [cortisol]}}{\text{urine [creatinine]}}$$

with [cortisol] in nmol/L and [creatinine] in mmol/L

Example: urine [cortisol] = 200 nmol/L; urine [creatinine] = 10 mmol/L

$$\text{UCCR} = \frac{200\ \text{nmol/L}}{10\ \text{mmol/L}} = \frac{200\ \text{nmol/L}}{10 \times 10^{6}\ \text{nmol/L}} = 20 \times 10^{-6}\ \text{(may be considered 20)}$$

(**18.2**)

II. Conclusions from published reports

A. Dogs with HAC
   1. A UCCR that is WRI is evidence that a dog does not have HAC, but increased UCCRs are frequently found in dogs without HAC. In other words, the UCCR has a high diagnostic sensitivity (> 90 % and approaching 100 %) for canine HAC (relatively few false negatives) and is useful as a screening test to help exclude HAC. However, it has shown insufficient positive predictive value and diagnostic specificity (relatively frequent false positives) to be considered a confirmatory test for HAC (Figure 18.4).[79,80] Note: Reported values for diagnostic specificity vary greatly because of variations in cortisol assays, creatinine assays, decision thresholds, diagnostic criteria, comparison groups, and environmental stress at the time of urine collection.

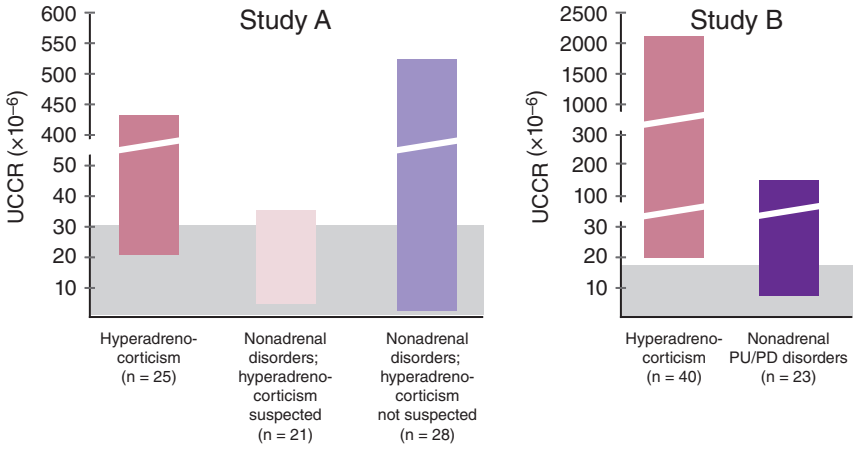


Figure 18.4. UCCRs in canine hyperadrenocorticism. The results of these two studies exemplify that when the UCCR is WRI, HAC is unlikely (but possible), whereas an increased UCCR is not specific for HAC.

- In study A,[79] the UCCRs from three groups of dogs were compared to those of a group of healthy dogs (*background grey region*; mean = 13×10^{-6}, $n = 31$) using urine samples collected in the veterinary hospital, which may decrease diagnostic specificity secondary to stress-induced hypercortisolemia.
  - Of 25 dogs with HAC (21 PDH and 4 FAN), 23 had increased UCCRs; the mean ratio was 103×10^{-6}.
  - Of 21 dogs with NAI (renal insufficiency, hepatic disease, pyelonephritis, hypothyroidism, bronchitis, and diabetes insipidus) but in which HAC was suspected, only one had an increased UCCR; the mean ratio was 16×10^{-6}.
  - In 28 dogs with moderate to severe NAI (gastrointestinal, renal, lower urinary tract, liver, neurologic, immune-mediated, cardiac, traumatic, and infectious diseases), 22 had increased UCCRs; the mean ratio was 83×10^{-6}.
- In study B,[80] the UCCRs from dogs with HAC (36 with PDH and 4 with FAN) and other polyuria/polydipsia disorders (diabetes insipidus, hypercalcemic disorders, hepatic disease, pyometra, and diabetes mellitus) were compared to the UCCRs found in healthy dogs (*background grey region*; mean = 6×10^{-6}, $n = 20$). Specifics regarding the dogs' locations at the time of voided urine collection were not provided. All 40 dogs with HAC had an increased UCCR but so did 18 of the 23 polyuria/polydipsia (PU/PD) dogs that did not have HAC. The mean UCCRs in the HAC and NAI groups were 338×10^{-6} and 43×10^{-6}, respectively.

Note: RIs vary with the analytical method, and decision thresholds should be based on studies involving only dogs for which testing is clinically indicated (HAC or HAC mimics), which was not the case in these studies.

2. UCCRs cannot reliably differentiate PDH from FAN, but the greatest ratios are usually associated with PDH, as is suppression of UCCR in conjunction with a HDDST.[81]
3. In a study involving 66 dogs with HAC and 87 dogs with clinical features similar to HAC, a UCCR decision threshold of 26.5×10^{-6} had the highest negative likelihood ratio, and a decision threshold of 161.2×10^{-6} had the highest positive likelihood ratio.[82] Thus, canine results below the lower threshold most likely do not have HAC and results above the upper threshold most likely do have HAC. Cutoffs vary with the methods; the upper reference limit for their methods was 30.8×10^{-6}.

B. Dogs with hypoadrenocorticism

1. The UCCR is low in a large majority of dogs with spontaneous hypoadrenocorticism when compared to dogs with illnesses considered to mimic hypoadrenocorticism. However, results can be equivocal, and decision thresholds should be determined or confirmed for each specific pair of urine cortisol and creatinine assays and analyzers.

2. In a retrospective study of 19 healthy dogs, 10 dogs with spontaneous hypoadrenocorticism (only 1 with normonatremia and normokalemia), and 18 dogs with disorders mimicking hypoadrenocorticism, UCCRs consistently differentiated hypoadrenocorticism from other illnesses, but 1 healthy dog had a ratio as low as those for hypoadrenocorticism, and a few others were close.[83]
3. In a prospective study of 41 dogs with spontaneous hypoadrenocorticism (2 without electrolyte evidence of hypoaldosteronism) and 107 dogs with NAI, the UCCR had 95 % diagnostic accuracy when urine [cortisol] was measured using a RIA (n = 75) and 100 % diagnostic accuracy when using a CLIA (n = 109) with empirically determined decision thresholds based on receiver operator characteristics.[84] Dogs with equivocal test results for diagnosing hypoadrenocorticism were not included.

C. Cats with HAC: In two studies at the same institution, UCCRs in all 13 cats with PDH were greater than UCCRs in all 42 healthy cats that were assessed.[85,86] A RIA was used to measure urine cortisol (corticoid) concentration.

D. Cats with hyperthyroidism: The UCCRs in 15 of 32 cats with hyperthyroidism were greater than the upper reference limit of the feline RI ($8.0 \times 10^{-6} - 42.0 \times 10^{-6}$; n = 45). For the hyperthyroid cats, the median UCCR was 37.5×10^{-6}, with two values above 100×10^{-6}.[87] The urine samples were collected at home from litter boxes containing nonabsorbent material. The greater UCCRs in hyperthyroid cats may reflect increased ACTH and cortisol production as reported in human hyperthyroidism,[88] and should not be considered reflective of pathologic HAC. Stress associated with the feline hyperthyroid state may also contribute. The median UCCR in cystocentesis samples from 16 hospitalized cats with NAI was about 5 times that of 31 healthy cats, though the ranges overlapped considerably.[89]

E. The UCCR did not appear to be a promising test for PPID in a study of 13 horses with PPID, 12 healthy horses, and 8 horses with dysautonomia syndrome.[90] UCCRs were similar between groups of ill horses and overlapped substantially with the UCCRs of healthy horses.

ADRENOCORTICOTROPIC HORMONE (ACTH) CONCENTRATION

I. Dogs and cats: Measuring [ACTH] can be helpful in differentiating PDH from FAN, and primary hypoadrenocorticism from secondary hypoadrenocorticism (Figure 18.5).

A. An [ACTH] is expected to be WRI or increased in animals with PDH, primary hypoadrenocorticism, ectopic production of ACTH,[45,46] food-dependent HAC,[47] and perhaps after stressful stimuli. Dogs with primary hypoadrenocorticism may also have an exaggerated increase in [ACTH] after an injection of corticotropin-releasing hormone.[91]

B. An [ACTH] is expected to be decreased in animals with FAN or secondary hypoadrenocorticism, after dexamethasone injections, and while animals are being given therapeutic doses of glucocorticoids (e.g., prednisone, prednisolone, or progestogens). Dogs with secondary hypoadrenocorticism have an inadequate increase in [ACTH] after an injection of CRH.[91]

C. The combination of plasma [ACTH] and results of adrenal ultrasonography can assist in differentiating PDH and FAN.[93] Adrenal glands of 21 dogs with PDH were of normal shape with similar size bilaterally, and each dog had a plasma [ACTH] $\geq$ 28 pg/mL (RI: 13–46 pg/mL). Each dog with FAN (6) had an adrenal mass and [ACTH] < 5 pg/mL. One dog had an [ACTH] < 5 pg/mL with ultrasonography results indicating PDH; retrospectively, the low [ACTH] was attributed to poor sample handling. Another dog had an [ACTH] of 76 pg/mL and ultrasonography results indicating an adrenal mass;

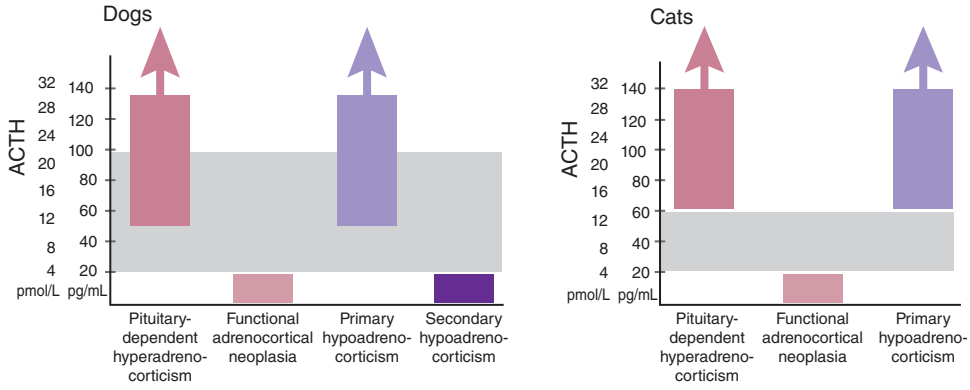


Figure 18.5. ACTH concentrations in adrenocortical disorders of dogs and cats.

- Dogs with PDH and primary hypoadrenocorticism have ACTH concentrations WRI or increased, whereas dogs with FAN and secondary hypoadrenocorticism are expected to have ACTH concentrations below lower reference limits. The background grey region represents the canine RI. Data for the graph were extracted from published concentrations.[91,92]
- Cats with PDH and primary hypoadrenocorticism are expected to have increased ACTH concentrations, whereas cats with FAN are expected to have decreased ACTH concentrations. The background grey region represents the feline RI. Data for the graph were extracted from published concentrations.[92]

Note: The arrows above the solid bars indicate that concentrations may be much greater than the values shown in the *y*-axes.

retrospectively, findings were attributed to a nonfunctional adrenocortical neoplasm or a pheochromocytoma. Other studies have shown a similar ability to differentiate FAN and PDH based on [ACTH].[94] Imaging of the pituitary gland may also be useful. A complicating factor is that pituitary and adrenal lesions can occur concurrently.

D. The [ACTH] in > 90 % of cats with PDH is increased or in about the upper third of the RI, whereas it is typically undetectable or low in cats with FAN.[40,41,95]

II. Horses

A. In horses (also ponies and donkeys), substantial seasonal (circannual) variation in pituitary secretion and [ACTH] requires seasonally adjusted RIs and decision thresholds appropriate for each geographical location and laboratory. Plasma ACTH concentrations are closely associated with the photoperiod, increasing with increasing daylight after the spring equinox and peaking at about the autumnal equinox before decreasing with decreasing hours of daylight.[96] Therefore, in the northern hemisphere, ACTH concentrations are greatest from August to October (peak in September) and lower from December through June, whereas in the southern hemisphere, they are greatest in February–April (peak in March) and lower in September. Increased autumnal pituitary secretion also increases plasma concentrations of α-melanocyte stimulating hormone and corticotropin-like intermediate lobe peptide. Measured ACTH concentrations include non-ACTH peptides with some assays.[33]

B. Plasma [ACTH] and ACTH responses are not uniform across breeds. Unstimulated [ACTH] is greater in Arabian horses, ponies, and donkeys compared to thoroughbred and standardbred horses, at least in autumn and with certain immunoassays (detection of cross-reactive peptides might contribute to greater differences in autumn).[44,96]

C. Although the [ACTH] in healthy horses is generally greater in the morning, samples can be collected at any time of day because the circadian variation is typically less than other individual and inter-individual variations, and no circadian rhythm was seen in horses with PPID.[97]

D. In a horse with clinical signs of PPID, an increased plasma [ACTH] compared to an appropriate RI or decision threshold for the laboratory, breed, and season is support for a pituitary gland adenoma or pituitary hyperplasia (PPID); lack of an increase helps exclude PPID. TRH stimulation testing may be helpful when results are equivocal.

III. Clinical use of [ACTH] is hampered by instability of ACTH and expensive dry-ice shipments.

PLASMA CORTISOL-TO-ACTH RATIO

I. Because of the negative feedback in the hypothalamic–pituitary–adrenal hormonal system, decreased production of cortisol by adrenal glands is expected to result in a high plasma [ACTH] (thus a low cortisol-to-ACTH ratio). Conversely, increased production of cortisol by an adrenal neoplasm is expected to result in a low plasma [ACTH] (thus a high cortisol-to-ACTH ratio). These relationships may be used to interpret individual hormone concentrations (i.e., expect a high [cortisol] with a high [ACTH] in PDH), but published attempts to use the ratios in diagnostic decisions have been limited.

A. In one published study, cortisol-to-ACTH ratios for 22 dogs with primary hypoadrenocorticism were consistently lower (range, 0.003–0.17 nmol/pmol or 3–170 as unitless ratios) than the ratios in 60 healthy dogs (0.79–175 nmol/pmol or 790–175,000 as unitless ratios).[98] The authors reported the ratios without adjusting for units (e.g., 0.003–0.17). Examples of the calculations are presented in Eq. 18.3.

Example using molar concentrations:

[cortisol] = 48 nmol/L (17 μg/L)

[ACTH] = 6.5 mmol/L (30 pg/L)

$$\text{Cortisol: ACTH ratio} = \frac{48\ \text{nmol/L}}{6.5\ \text{pmol/L}} = \frac{7.4\ \text{nmol cortisol}}{\text{pmol ACTH}} = \frac{7400\ \text{pmol cortisol}}{\text{pmol ACTH}} = 7.4 \times 10^3$$

The ratio reported in the article for these data would have been 7.4. (**18.3a**)

Same example using the weight/volume concentrations:

$$\text{Cortisol: ACTH ratio} = \frac{17\ \mu\text{g/L}}{30\ \text{pg/L}} = \frac{0.57\ \mu\text{g cortisol}}{\text{pg ACTH}} = \frac{570,000\ \text{pg cortisol}}{\text{pg ACTH}} = 570 \times 10^3 \quad (\textbf{18.3b})$$

A ratio with these units was not reported in the article.

B. Similar results were found in a small retrospective study that included 19 dogs with NAI and clinical signs or laboratory data suggestive of hypoadrenocorticism; the cortisol to ACTH ratios in the 15 dogs with hypoadrenocorticism (9 of which lacked electrolyte abnormalities) were consistently less than those in healthy dogs or dogs with NAI.[99]

C. In a prospective study comparing results for 23 dogs with hypoadrenocorticism, 79 dogs with NAI mimicking hypoadrenocorticism, and 30 healthy dogs, no healthy dogs and only 1 dog with NAI had a cortisol to ACTH ratio as low as any of those for dogs with

hypoadrenocorticism.[100] The one overlapping value was caused by a very high [ACTH] that was not explained despite 1.5 yr of follow-up.

II. Because of the lack of agreement among hormone assays, interpretive guidelines should be developed for each set of cortisol and ACTH assays. The cortisol-to-ACTH ratios may be helpful in differentiating primary from secondary hypoadrenocorticism, but a low [ACTH] alone may be sufficient to identify secondary hypoadrenocorticism. The ratio also might have value in differentiating forms of HAC. However, the special sample handling required for ACTH samples and the associated expenses limit the use of cortisol-to-ACTH ratios.

SUPPRESSION AND STIMULATION TESTS FOR ASSESSING ADRENOCORTICAL RESPONSES

I. Dogs[52]
   A. Low-dose dexamethasone suppression test (LDDST): a screening test for PDH and FAN
      1. Procedure
         a. A pre-dexamethasone blood sample is collected and processed.
         b. Dexamethasone is given at 0.01–0.015 mg/kg IV (some use IM); 1.00 mg/kg dexamethasone sodium phosphate is equivalent to 0.76 mg/kg dexamethasone.
         c. Post-dexamethasone samples are processed after collections at 4 h and 8 h (some use 3 h and 8 h, 4–6 h and 8 h, or just 8 h).
         d. Pre-dexamethasone and post-dexamethasone plasma or serum samples are submitted for measurement of [cortisol].
      2. Expected results (Figure 18.6)
         a. In healthy animals, dexamethasone is expected to reduce the secretion of CRH and ACTH for several hours and thus decrease release of cortisol from the adrenal glands. Because the half-life of cortisol is < 2 h, plasma [cortisol] is expected to be decreased dramatically (i.e., suppressed) at 4 h and 8 h (Figure 18.6A). Values are usually inadequately suppressed at 8 h with PDH and FAN. However, if HAC is caused by PDH or ectopic production of ACTH, then a low dose of dexamethasone may not suppress release of ACTH by neoplastic pituitary cells, and thus cortisol production from hyperplastic adrenal glands may not decrease.
         b. Although medical decision thresholds depend on the assay, many veterinarians have interpreted a post-dexamethasone [cortisol] < 1.4 μg/dL (< 39 or 40 nmol/L) to indicate there was adequate suppression of cortisol secretion. This decision threshold appears to have been calculated from RIA data collected from just 22 healthy dogs; it represented the mean concentration (0.5 μg/dL) plus three standard deviations (3 × 0.3 = 0.9 μg/dL) at 8 h.[101,102] Using a more contemporary assay for cortisol (a CLIA), the same 8-h decision threshold (39 mol/L, 1.4 μg/dL) was derived from a receiver operator characteristic curve based on 24 dogs with PDH, 3 with FAN, and 30 healthy dogs.[105] However, a decision threshold of < 1 μg/dL (27.6 nmol/L) has been used for the same CLIA, and a change in the antibody used for the CLIA has yielded substantially different results that may require use of a different decision threshold.[106] Results vary with the procedure, antibodies, and instrument.
         c. Partial suppression can be considered present if the post-dexamethasone [cortisol] in the 4 h or 8 h samples is above the cutoff for suppression but < 50 % of the pre-dexamethasone concentration.

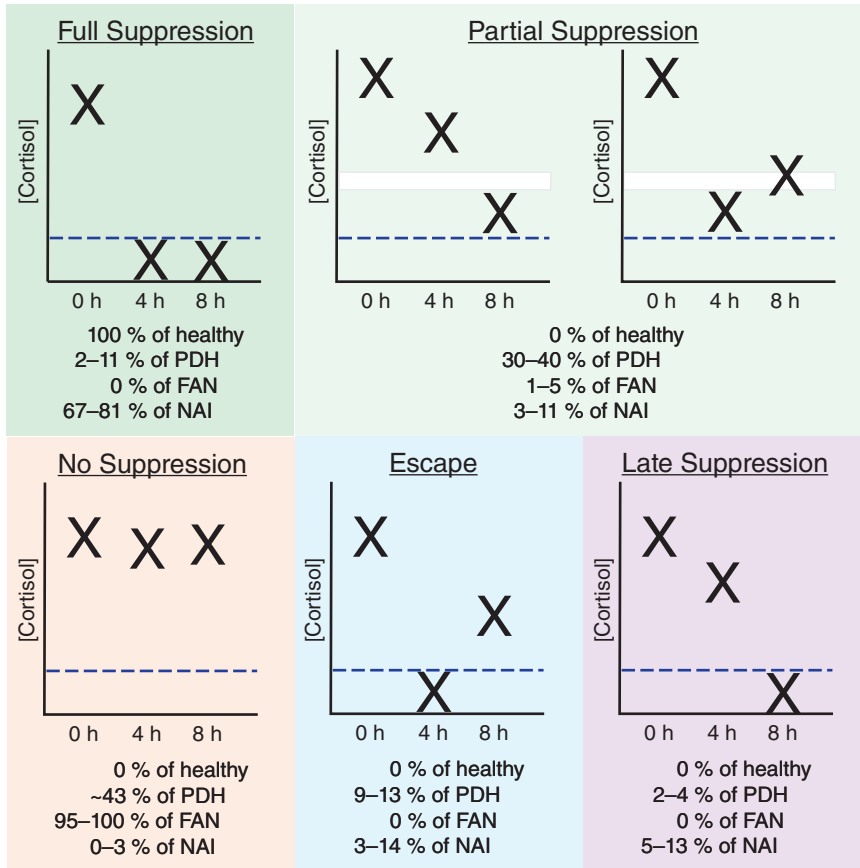


Figure 18.6. Patterns and interpretation of LDDST responses in dogs. Expected findings are provided under the graph for each pattern.[a] Results at 0 h may be WRI or increased for dogs with PDH, FAN, or NAI (RI at 0 h not shown).

Full suppression: The [cortisol] at 4 h (or 3 h) and 8 h decreases to below the laboratory decision threshold (*dashed blue line*), e.g., < 1 μg/dL (28 nmol/L) or < 1.4 μg/dL (39 nmol/L). This pattern is expected in health.

- Interpretation: FAN is very unlikely and PDH is unlikely. NAI is very likely if clinical findings support disease.

Partial suppression: The [cortisol] decreases to < 50 % of the pre-dexamethasone (0 h) [cortisol] in one or both subsequent samples but stays above the decision threshold; the white bars in the graphs for partial suppression indicate the range of values that are 50 % of the range at 0 h (i.e., distance from bottom to top of the 0-h "X").

- Interpretation: PDH is most likely, but FAN and NAI cannot be excluded.

No suppression: The [cortisol] is at or above the decision threshold at 4 h (or 3 h) and 8 h.

- Interpretation: HAC is very likely, either FAN or PDH. Almost all dogs with FAN have this pattern, but it occurs in only about half of dogs with PDH. It occurs in a few dogs with NAI.

Escape: The [cortisol] is below the decision threshold at 4 h (or 3 h) but not at 8 h.

- Interpretation: PDH or NAI may be present; the pattern is not expected with FAN or good health.

Late suppression (inverse pattern): The [cortisol] is at or above the decision threshold at 4 h (or 3 h) but below it at 8 h.

- Interpretation: PDH or NAI may be present; the pattern is not expected with FAN or good health.

[a] Sources of data for graphed responses: Responses for healthy dogs are those expected based on common decision limits.[101,102] Data for dogs with PDH, FAN, and NAI were extracted from published results.[103,104] In both studies, suppression was defined as a post-dexamethasone [cortisol] < 1.0 μg/dL. Causes of NAI varied between studies (e.g., acute disorders, untreated diabetes mellitus, and dogs presented for obesity workups were excluded in one study), but all dogs had a LDDST as part of the clinical evaluation. Differences in exclusion and inclusion criteria for dogs with NAI were likely a major cause of differences in response patterns for this group.

d. In some cases, the 4-h post-dexamethasone [cortisol] is below the decision threshold, but the 8-h post-dexamethasone [cortisol] is above the decision threshold. This escape pattern is supportive of PDH when other clinical findings are also supportive, but escape occurs with NAI often enough that additional testing may be needed to reach a firm diagnosis of HAC.[103,107]
e. If HAC is caused by FAN, then low-dose dexamethasone is not expected to decrease the secretion of cortisol, although mild decreases could occur, particularly with a low pre-dexamethasone [cortisol]. This is because of analytical imprecision or variations in cortisol secretion by neoplastic cells, cortisol binding to protein, or plasma cortisol clearance. The LDDST has high diagnostic sensitivity for FAN; suppression by ≥ 50 % rarely occurs with FAN, and even 30–50 % suppression is unlikely.[107]
f. Suppression may not be adequate in dogs with NAI. Consequently, the LDDST has only moderate (e.g., ~70 %) diagnostic specificity for HAC.[107]
g. In two dogs receiving phenobarbital therapy, cortisol concentrations were not suppressed adequately, possibly because phenobarbital's effects on clearance of synthetic steroids enhance dexamethasone's clearance rate.[108] However, phenobarbital therapy did not affect results of the LDDST in other dogs.[107,108] Other drugs that may increase (e.g., carbamazepine) or decrease (e.g., diltiazem, cimetidine) dexamethasone metabolism have the potential to decrease and increase, respectively, the dexamethasone-induced suppression of [cortisol].[5]
h. Too few cases of ectopic ACTH production have been reported to establish a pattern, but there should be lack of suppression of [cortisol].

B. High-dose dexamethasone suppression test (HDDST), a test to help differentiate PDH from FAN in dogs with HAC
1. Procedure: the same as for the LDDST except that dexamethasone is given at 0.1 mg/kg IV (some use IM)
2. Expected results
a. In healthy animals, dexamethasone is expected to dramatically reduce plasma [cortisol] at 4 h, and concentrations should remain decreased in the 8 h samples.
b. If HAC is caused by PDH, then high-dose dexamethasone usually causes suppression of ACTH release by neoplastic pituitary cells and thus reduces cortisol production from hyperplastic adrenal glands.
c. If HAC is caused by FAN, then high-dose dexamethasone is not expected to decrease the secretion of cortisol (no suppression). Even though suppression criteria are not fulfilled, cortisol concentrations may fluctuate during the testing because of analytical variation or variations in cortisol secretion by neoplastic cells, cortisol binding to proteins, or plasma cortisol clearance.
d. If a HDDST is done in a dog with NAI, then cortisol production is expected to be decreased sufficiently to indicate a suppression of ACTH secretion.
e. Too few cases of ectopic ACTH production have been reported to establish a pattern, but lack of suppression of [cortisol] is expected.
3. The HDDST can be paired with UCCR assessment to help differentiate PDH (UCCR suppresses to < 50 % of baseline) from FAN.[81]

C. Interpretation of dexamethasone suppression tests
1. Expected patterns of LDDST and HDDST results for individual dogs
a. In healthy dogs, [cortisol] is suppressed with both tests (4 h and 8 h samples).

b. If HAC is caused by PDH, then [cortisol] is usually suppressed with the HDDST and often with the LDDST. The HDDST therefore provides evidence for PDH in a subset of dogs for which the LDDST yields no suppression and therefore cannot differentiate PDH from FAN.
c. If HAC is caused by FAN or ectopic production of ACTH, then lack of [cortisol] suppression is expected in nearly all post-dexamethasone samples.
d. In dogs with NAI, there may or may not be cortisol suppression with the LDDST. The [cortisol] is expected to be suppressed with the HDDST.

2. Table 18.4 provides examples of cortisol concentrations from dexamethasone suppression tests. Figure 18.6 can be used a guide. Best interpretations are made when results are interpreted in the context of historical and physical findings.

D. Adrenocorticotropic hormone (ACTH) stimulation (response) test
1. Exogenous ACTH is the stimulant; [cortisol] is assessed for the response.
2. Different ACTH products, routes of administration, and doses have been used, and results of some have been directly compared. Results and interpretation are generally similar,[109–111] but some differences are recognized.[107,112] Only validated methods and products should be used. Laboratories can be consulted for information about current local protocols and preferences.
3. Prior systemic or topical treatment (including otic and ocular) with certain glucocorticoids may suppress responses in the ACTH stimulation test for 2 wk (e.g., prednisolone) or 4 wk (long-acting glucocorticoids) after cessation of treatment. Also, many glucocorticoids (e.g., cortisone, hydrocortisone, prednisolone) other than dexamethasone interfere with the cortisol assay and should not be administered within 24 h of testing.
4. Administration of butorphanol to healthy dogs 90 min before ACTH stimulation testing significantly increased pre- and post-ACTH [cortisol] enough to recommend avoiding its use prior to such testing, but dogs with adrenal disease were not tested.[113]
5. Basic procedure (may begin at any time of day)
a. A sample for pre-ACTH [cortisol] is typically collected, but only the post-ACTH [cortisol] is needed when evaluating only for PDH.[114]
b. ACTH is administered to stimulate cortisol production. This is typically cosyntropin (tetracosactrin), a synthetic ACTH; different doses are used, 5 μg/kg IV being common. ACTH gels are administered IM at 2.2 IU/kg.
c. A post-ACTH sample is collected 1 h later (and 2 h for gels) to assess for a cortisol response.
6. Expected results (Figure 18.7) (Specific patient results should be interpreted by using RIs and interpretation guidelines provided with results.)
a. Healthy dogs
(1) ACTH should stimulate the production and release of cortisol from the adrenal cortices.
(2) Expected pre-ACTH and post-ACTH concentrations of cortisol vary among published reports and reference laboratories, but post-ACTH RIs are substantially higher than the pre-ACTH RIs and do not overlap.
b. Hypoadrenocorticism
(1) Dogs with primary, secondary, or iatrogenic adrenocortical hypoplasia or atrophy are expected to have fewer or no responsive cells, and thus the post-ACTH [cortisol] is inadequately increased or not increased at all.

Table 18.4. Interpretation of example dexamethasone suppression test results for dogs

	LDDST			HDDST		
	Cortisol (μg/dL)[a]			Cortisol (μg/dL)		
	Pre[b]	4 h post[b]	8 h post	Pre	4 h post	8 h post
In health	0.5–6.0	< 1.4[c]	< 1.4	0.5–6.0	< 1.4	< 1.4
Dog 1[d]	4.5	3.0	3.5	3.5	3.3	2.9
Dog 2[e]	7.0	1.0	3.8	6.2	0.8	0.6
Dog 3[f]	5.0	0.9	0.6	5.5	0.5	0.3
Dog 4[g]	6.5	3.2	2.8	4.5	0.8	0.4
Dog 5[h]	0.2	0.1	0.1	—	—	—

[a] Conversion to SI units: μg/dL × 27.6 = nmol/L (round to nearest 10)
[b] Pre, pre-dexamethasone [cortisol]; post, post-dexamethasone [cortisol]
[c] For these data, the decision threshold for suppression is 1.4 μg/dL.
[d] Dog 1 had either PDH or FAN. With the LDDST, inadequate suppression indicates PDH, FAN, or NAI. With the HDDST, inadequate suppression is more likely in FAN but can be found with PDH; adequate suppression is expected with NAI.
[e] Dog 2 had adrenal hyperplasia caused by PDH or a hyperresponsive adrenal gland because of NAI. With the LDDST, 4 h suppression and then escape at 8 h suggests PDH or nonadrenal disorders. With the HDDST, suppression can be found with PDH and is expected with NAI but is not consistent with FAN.
[f] Dog 3 had PDH or NAI (or was tested despite being healthy). With the LDDST, suppression can be found with health, PDH, and NAI. With the HDDST, suppression can be found with health, PDH, and NAI. Suppression is not expected with FAN.
[g] Dog 4 had adrenal hyperplasia caused by PDH or NAI. With the LDDST, partial suppression at 4 h and 8 h could reflect PDH, NAI, or possibly FAN. With the HDDST, suppression can be found with PDH and is expected with NAI, but it is not supportive of FAN.
[h] Dog 5 had a hypoadrenal state. With the LDDST, the pre-dexamethasone hypocortisolemia indicates a hypoadrenal state that could be primary, central, or iatrogenic hypoadrenocorticism. The HDDST is not useful in such cases.

(2) Pre-ACTH and post-ACTH cortisol concentrations below or not exceeding approximately the bottom quartile of the pre-ACTH RI for [cortisol] support adrenocortical atrophy, destruction, or hypoplasia, but could also occur with (i) an adrenal neoplasm that does not secrete cortisol or (ii) preanalytical problems including insufficient ACTH dose or potency (e.g., storage, protocol, or administration problem) and mistiming of sample collection.

c. HAC

(1) About 80–90 % of dogs with PDH and about 50 % of dogs with FAN have exaggerated responses to ACTH that reflect either bilateral adrenocortical hyperplasia or responsive neoplastic cells. Dogs with ectopic production of ACTH also are expected to have an exaggerated increase in [cortisol] because of the presence of bilateral adrenocortical hyperplasia.

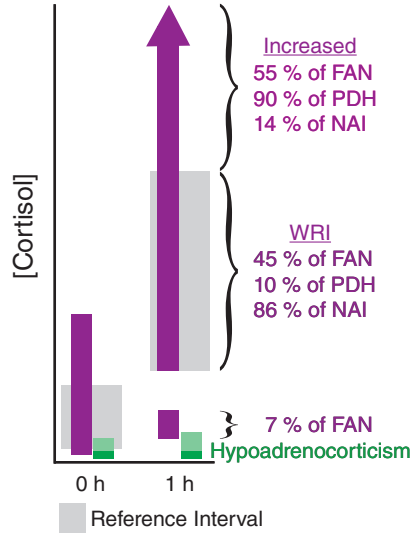


Figure 18.7. Responses from canine ACTH-stimulation tests. Assays and criteria used to determine appropriate (WRI), inadequate (below lower reference limit), or exaggerated (greater than upper reference limit) responses to ACTH stimulation varied among publications used for this figure. For each set of extracted data, the authors' criteria were used. The cortisol results are shown as bars representing the span of data relative to the grey regions, which represent RIs for pre- or post-ACTH [cortisol].

- Spontaneous hypoadrenocorticism (*included in green bars*): Most dogs had a decreased pre-ACTH [cortisol] (*dark green portion of bar*), but some were in the lower portion of the RI (*lighter green*). Responses to ACTH stimulation were inadequate, with most post-ACTH cortisol concentrations remaining below the pre-ACTH lower reference limit and a minority increasing mildly. The 225 cases included 220 cases of primary spontaneous hypoadrenocorticism and 5 cases of secondary hypoadrenocorticism.[91]
- Iatrogenic HAC (*included in green bars*): Adrenocortical atrophy resulted in no (16) or reduced (12) responses to ACTH stimulation in all 28 cases.[115] The mean pre-ACTH [cortisol] was at the lower reference limit, and the mean post-ACTH [cortisol] was about twice that.
- PDH and FAN cases (*included in purple bars*): In 90 % of 82 PDH cases and in 55 % of 42 FAN cases, there were exaggerated responses to ACTH stimulation; responses were not exaggerated in 10 % of PDH cases and in 45 % of FAN cases.[101,116–121] In 7 % of FAN cases, response to ACTH stimulation was decreased and sometimes minimal; the pre-ACTH [cortisol] was relatively low in these dogs.
- NAI (*include in purple bars*): Responses to ACTH stimulation were exaggerated in 14 % of 59 dogs and WRI in the other 86 %.[117] Although HAC was not suspected in these dogs, the data show nonspecificity of exaggerated ACTH responses. In another study, 10 (34 %) of the 29 dogs with disorders mimicking HAC had exaggerated ACTH responses (data not included).[122]

General summary

- Little or no response to ACTH supports spontaneous hypoadrenocorticism or, if a dog has clinical signs of hypercorticism, either (i) iatrogenic hypoadrenocorticism or (ii) FAN with impaired cortisol production but often production of other steroid hormones.
- An exaggerated response to ACTH supports HAC (PDH or FAN) but could also occur with NAI, so more information is needed to reach a diagnosis.
- If the response to ACTH is WRI, HAC or NAI could be present.

(2) Some adrenocortical neoplasms may not respond because the neoplastic cells have defects in cortisol production (or possibly receptors or signaling pathways); adrenal neoplasia should be suspected in dogs with clinical signs of HAC, no recent adrenosuppressive steroid treatment, and little or no cortisol response to ACTH.

(3) Dogs with iatrogenic hypercorticism from chronic glucocorticoid administration have little or no response.

(4) Expected responses may not occur if there were problems with the ACTH or procedure.

d. NAI

(1) Some dogs with disorders other than PDH or FAN have exaggerated responses to ACTH. Typically, these animals are thought to have stress-induced hypercortisolemia. They may or may not have clinical signs suggestive of HAC.

(2) With critical illness, the response to exogenous ACTH may instead be blunted, with just a small increment of increase in [cortisol] (a small *delta cortisol*) associated with a baseline [cortisol] that may be decreased, WRI, or increased. This has been labeled *critical illness-related corticosteroid insufficiency (CIRCI)* or relative adrenal insufficiency; it has been reported in dogs with systemic inflammatory response syndrome and bacterial sepsis[54,123] as well as in sick foals.[124]

(a) The suppressed response of [total cortisol] to ACTH may reflect a decreased adrenal reserve, particularly with prolonged illness when decreased cortisol clearance and increased [free cortisol] may suppress ACTH and lead to adrenocortical atrophy.[69]

(b) However, increases in [free cortisol] may be masked by decreased protein-bound fractions, in which case use of the ACTH stimulation test may be misleading in critically ill patients.[69] This may occur because of hypoalbuminemia or a decreased plasma concentration of cortisol-binding globulin, a negative acute phase protein in at least rats and people.[56]

e. After o,p′DDD (mitotane, Lysodren) treatment

(1) o,p′DDD is an adrenocorticolytic compound that is used to treat PDH and FAN. The compound damages the zona fasciculata and the zona reticularis.

(2) ACTH stimulation tests can be used to assess destruction of adrenocortical tissue. Typically, the goal of o,p′DDD treatment is to adequately reduce the post-ACTH [cortisol] but not cause enough destruction to create a hypoadrenocortical state.

f. After trilostane treatment

(1) Trilostane, which is an alternative to o,p′DDD for treatment of PDH, reduces steroid synthesis by inhibiting 3β-hydroxysteroid dehydrogenase and thus reduces the synthesis of cortisol, aldosterone, and other steroid hormones. Trilostane reduces [cortisol] in dogs with PDH and also reduces [aldosterone], but to a lesser degree.[71]

(2) ACTH stimulation tests can be used to monitor the effectiveness of trilostane treatment. If a dog has continued clinical signs of HAC and the post-ACTH [cortisol] is considered too high (e.g., > 9 μg/dL), then the dosage can be increased. If the dog develops persistent clinical signs suggestive of hypoadrenocorticism and the post-ACTH [cortisol] is low (e.g., < 1.0 μg/dL),

then treatment can be discontinued.[125] Treated dogs can develop hypocortisolemia and suppressed responses to ACTH without clinical signs of hypoadrenocorticism.[126] Results of ACTH stimulation testing 2–6 h after trilostane administration do not align well with owner-reported clinical signs and satisfaction.[127]

II. Cats
  A. Low-dose dexamethasone suppression test (LDDST)
    1. Procedure: Basically, the procedure is the same as for the canine LDDST except that dexamethasone is given at a dose of 0.1 mg/kg IV (as used for the canine HDDST, but generally now considered a low dose for the test in cats). Use of the canine low dose (0.01 or 0.015 mg/kg) is not recommended for cats because it does not suppress [cortisol] in about 15–20 % of healthy cats and therefore has poor diagnostic specificity for feline HAC.
    2. Expected results[41,95] (Specific patient results should be interpreted by using RIs and interpretation guidelines provided with results.)
      a. In almost all healthy cats, the 4-h and 8-h cortisol concentrations are suppressed and therefore < ~1.0 μg/dL (< ~30 nmol/L).
      b. In over 90 % of cats with HAC, the 4-h and 8-h cortisol concentrations are not suppressed and therefore ≥ ~1.4 μg/dL (≥ ~40 nmol/L); a small minority show suppression at 4 h but not at 8 h (escape pattern). A post-dexamethasone [cortisol] of ~1.0–1.4 μg/dL (~30–40 nmol/L) is equivocal. As in dogs, suppression may not be evident in some cats with NAI.
      c. Cats with FAN are expected to lack suppression at 4 h and 8 h, and the 4-h and 8-h cortisol concentrations are expected to be at least 50 % of the baseline [cortisol].
      d. In contrast, a few cats with PDH have an escape pattern (suppression at 4 h but not 8 h), and approximately 50 % of cats with PDH have more than a 50 % decrease in [cortisol] (to < 50 % of baseline, but not below the decision threshold for suppression) at 4 h, 8 h, or both. These patterns support PDH rather than FAN in a cat with HAC.
    3. Dexamethasone suppression can be paired with UCCR assessment as a screening test for HAC and to help differentiate PDH from FAN.
      a. Procedure: After at-home collection of urine for two consecutive days, 0.1 mg/kg dexamethasone is administered orally three times at 8-h intervals followed by collection of a third urine sample on day 3.
      b. Interpretation: HAC is unlikely if the pre-dexamethasone UCCR is not increased. If it is increased, there are clinical signs of HAC, and the UCCR in the day-3 urine is < 50 % of the pre-dexamethasone UCCR, the findings support PDH.[85,86] Suppression also occurs in healthy cats.
  B. High-dose dexamethasone suppression test (HDDST), a test to help differentiate PDH from FAN in cats with HAC
    1. Procedure
      a. The protocol is the same as with the canine HDDST, but 1 mg/kg dexamethasone is generally used.
      b. Cats may escape the suppressive effects of dexamethasone faster than dogs, so suppression may be seen only if samples are collected at 2 h, 4 h, and 6 h after dexamethasone.[128]

2. Expected results for the 1.0 mg/kg dose[41,95]
   a. Expected findings are based on relatively few published cases of these uncommon disorders. Specific patient results should be interpreted by using RIs and interpretation guidelines provided with results.
   b. When suppression is defined as at least a 50 % decrease in [cortisol] from baseline at either 4 h or 8 h after dexamethasone administration, suppression is not expected in cats with FAN and occurs in about 50 % of cats with PDH. Lack of suppression does not differentiate these conditions.

C. Adrenocorticotropic hormone (ACTH) stimulation (response) test
   1. Procedure (may begin at any time of day)
      a. A sample for pre-ACTH [cortisol] is collected.
      b. Synthetic ACTH (tetracosactide, cosyntropin) is given IV at 5 µg/kg or 0.125 mg per cat. ACTH gel is used at 2.2 IU/kg IM.
      c. Post-ACTH samples are collected 60 min later and potentially other times up through 180 min, depending on the procedure preferred by the laboratory doing the analysis.
   2. Expected results[95]
      a. Specific patient results should be interpreted by using RIs and interpretation guidelines provided with results.
      b. ACTH should stimulate the production and release of cortisol from the adrenal cortices.
      c. Compared to appropriate pre-ACTH and post-ACTH RIs, only about 30–50 % of cats with spontaneous HAC have an exaggerated response, and some cats with NAI may also.
      d. Cats with primary hypoadrenocorticism have decreased or low-normal pre-ACTH cortisol concentrations with little or no increase in [cortisol] after ACTH administration.[60,61] Similar findings occur in cats with iatrogenic hypercorticism, but clinical findings differ, and endogenous ACTH concentrations would be high with the former and low with the latter.

III. Horses

A. Dexamethasone suppression test
   1. Overnight method (check laboratory for preferences)[7,129]
      a. A sample for pre-dexamethasone [cortisol] is collected at 4–6 p.m.
      b. Dexamethasone is given at 40 µg/kg (20 mg/1100 lb) IM.
      c. Post-dexamethasone samples are collected at about noon the next day (18–20 h after dexamethasone), or two samples are collected at 15 h and 19 h.
   2. Expected results (Note: Specific patient results should be interpreted by using RIs and interpretation guidelines provided with results.)
      a. Healthy horses: Negative feedback on the pars distalis (but not the pars intermedia) causes adequate suppression resulting in a post-dexamethasone [cortisol] < 1.0 µg/dL (< 28 nmol/L) during seasons of lower ACTH production. Higher decision thresholds, if available, would be appropriate for autumnal samples, particularly in breeds with greater ACTH concentrations.[96,130]
      b. PPID: Inadequate suppression of ACTH production because of pars intermedia hyperplasia or neoplasia may result in a post-dexamethasone [cortisol] > 1.0 µg/dL

(> 28 nmol/L) during seasons of lower ACTH production. Use of this decision threshold for autumnal samples and certain breeds may yield false negative results for suppression (false positive for PPID) because of the greater pituitary secretion at that time. Marked suppression in the autumn supports exclusion of PPID.

B. Cortisol concentrations during a 24-h period after dexamethasone (40 μg/kg)[7]
   1. In 34 healthy horses, cortisol concentrations were decreased 8–24 h after dexamethasone. All had concentrations < 1.0 μg/dL at 20–24 h after dexamethasone.
   2. In 52 horses with PPID, the maximal suppression was seen 8–12 h after dexamethasone. All had cortisol concentrations > 1.0 μg/dL at 20–24 h after dexamethasone.
   3. Seasonal variation: Concurrent with the seasonal variation in [ACTH], results of the equine dexamethasone suppression test were different in January compared with those in September.[130] Seasonal RIs may be needed for appropriate interpretation of test results.

C. Adrenocorticotropic hormone (ACTH) stimulation (response) test
   1. Because of substantially overlapping [cortisol] results for healthy horses and horses with PPID, the ACTH stimulation test is not recommended as a diagnostic test for equine PPID.[7,22] It may be used to evaluate adrenal dysfunction associated with illness.
   2. Multiple products and protocols have been used, including ACTH gels (1 IU/kg IM) with post-ACTH sample collection 1–4 h later, and synthetic ACTH at 0.1–1 μg/kg IV with post-ACTH sample collection at 0.5–2 h later. The protocol should match that for which a laboratory has determined RIs.
   3. In healthy horses, the post-ACTH [cortisol] is usually about 2–4 times the pre-ACTH [cortisol].[7,22,131]

D. Thyrotropin-releasing hormone (TRH) stimulation (response) test[44]
   1. TRH stimulates secretion of ACTH (and α-melanocyte-stimulating hormone, corticotropin-like intermediate lobe peptide, and β-endorphin) from the pars distalis or pars intermedia of the pituitary gland. An exaggerated response to TRH supports the presence of a pituitary gland adenoma or pituitary hyperplasia (usually PPID).
   2. After collecting a baseline blood sample, 1 mg of TRH is administered IV (0.5 mg in ponies), and a second sample is collected either precisely 10 min later or 30 min later, when concentrations are dropping from the peak. Collecting a post-TRH sample after 9 min or 11 min rather than 10 min can alter interpretation;[132] less change occurs per minute after 30 min, so the later sample results are less impacted by minor errors in timing.
   3. When insulin-sensitive mares were sedated with detomidine alone or detomidine plus butorphanol 10 min prior to TRH stimulation testing, the baseline [ACTH] was not affected, but there was a significantly exaggerated increase in [ACTH] after 5 min, and insignificant but numerically increased responses at 10 min and 30 min (less so).[133]
   4. An exaggerated response is indicated by a plasma [ACTH] that exceeds a decision threshold appropriate for the collection time, laboratory, season, and breed. Incremental and percentage responses in spring and autumn can be very different from each other. Lack of an exaggerated response in the fall may be particularly useful to exclude PPID.

COMBINED DEXAMETHASONE SUPPRESSION–ADRENOCORTICOTROPIC HORMONE (ACTH) STIMULATION (RESPONSE) TEST

The major advantage of the combined test is that it combines adrenal assessment into one diagnostic procedure, and thus only one set of samples is submitted to a reference laboratory. The major disadvantage in dogs is that it does not have the same diagnostic power as the combination of LDDST and HDDST done individually. It is a screening test for HAC.

I. Canine method[102]
   A. A sample for a baseline [cortisol] is collected, and then dexamethasone is administered (0.1 mg/kg IV). A post-dexamethasone sample is collected 2–4 h later, and then the ACTH stimulation test is done as described in Suppression and Stimulation Tests, Section I.D. All samples are submitted for measurement of [cortisol].
   B. The dexamethasone dose is the same as that used in the HDDST, but the post-dexamethasone sample is drawn only at 2–4 h and thus does not assess the possible escape in the 8 h sample of the HDDST. Lack of suppression supports HAC, but suppression occurs in some dogs with PDH (see HDDST above).
   C. An exaggerated ACTH response supports HAC but does not reliably differentiate PDH and FAN.

II. Feline method[134]
   A. A sample for a baseline [cortisol] is collected, and then dexamethasone is administered (0.1 mg/kg IV). A post-dexamethasone sample is collected 2–4 h later, and then the ACTH stimulation test is done as described in Suppression and Stimulation Tests, Section II.C. All samples are submitted for measurement of [cortisol].
   B. In two reported cases of feline PDH, there was inadequate suppression after dexamethasone administration, and there were exaggerated responses to ACTH.[134,135]
   C. In one study involving 35 cats with NAI (17 diabetic and 18 nondiabetic) and 19 healthy cats, results were very similar among groups.[136]

III. Equine method[137]
   A. A sample for a baseline [cortisol] is collected, and then dexamethasone is administered (10 mg IM at 9 a.m.). A post-dexamethasone sample is collected 3 h later, and then cosyntropin is administered IV (1 mg = 100 IU). Then, a post-ACTH sample is collected 2 h later. All samples are submitted for measurement of [cortisol].
   B. There has been limited use of the procedure in horses. In one study, the combined test did not reliably differentiate six healthy horses from six horses with PDH.[7]

ALDOSTERONE CONCENTRATION

I. Because ACTH stimulates the release of cortisol and aldosterone, aldosterone concentrations before and after ACTH administration reflect adrenal function. The [aldosterone] is typically decreased with clinical hypoadrenocorticism and increased with primary and secondary hyperaldosteronism.

II. Primary hyperaldosteronism: This disorder is rare in dogs and uncommon in cats. In dogs and cats, it is usually caused by an aldosterone-secreting adrenal gland neoplasm, but idiopathic bilateral hyperplasia of the zona glomerulosa has been reported.

A. Dogs
   1. Presenting complaints in dogs with primary hyperaldosteronism have commonly included muscle weakness, polyuria, and polydipsia. Hypokalemia is the most common routine laboratory abnormality. An adrenal mass has been evident in most cases, and contralateral atrophy may be present.
   2. Aldosterone assays have varied, and the number of reference individuals has been limited, but reported cases of hyperaldosteronism have been supported by increased plasma [aldosterone] pre-ACTH stimulation, post-ACTH, or both.
      a. In one dog, basal aldosterone concentrations were 740 and 840 pmol/L compared to 30–210 pmol/L in 12 healthy dogs; decreased [renin] was documented, thus supporting primary hyperaldosteronism, and results of a LDDST were WRI.[138]
      b. A bichon frise with a metastatic adrenal carcinoma had increased pre- and post-ACTH aldosterone concentrations of 1747 pmol/L and > 3329 pmol/L, respectively, compared to pre- and post-ACTH RIs of 14–957 pmol/L and 197–2103 pmol/L, respectively.[139] Pre- and post-ACTH aldosterone concentrations decreased following adrenalectomy, increased to > 3329 pmol/L with progression of the neoplasm, and decreased again with resection of metastases. The neoplasm also appeared to secrete excessive corticosterone based on an increased post-ACTH [corticosterone] compared to the response of six healthy greyhounds, and the dog had contralateral adrenal atrophy and a suppressed cortisol response to exogenous ACTH, presumably because of corticosterone-induced suppression. The source of the RIs was not provided, but the same pre-ACTH RI was provided with results generated at a commercial laboratory for a dog with primary hyperaldosteronism, concurrent lymphoma, and an aldosterone concentration of 1949 pmol/L. This dog's adrenal neoplasm was positive for cytoplasmic aldosterone by immunohistochemistry.[140]
      c. The aldosterone response to ACTH was marked in a beagle with a metastatic adrenocortical carcinoma, increasing from 3601 pmol/L before ACTH to > 15,000 pmol/L and > 20,000 pmol/L 30 min and 60 min after ACTH administration, respectively; the baseline, 30-min, and 60-min aldosterone concentrations for six healthy beagles were 211–335 pmol/L, 729–1391 pmol/L, and 418–1000 pmol/L, respectively.[141] The dog also had increased baseline and post-ACTH concentrations of cortisol and corticosterone.
      d. In one report, the [aldosterone] in each of three dogs was > 3000 pmol/L, but the RI was not provided;[142] this can be compared to the preceding data and the pre-ACTH aldosterone concentrations in healthy dogs in Table 18.5.

B. Cats
   1. As in dogs, most cases of primary hyperaldosteronism have been caused by adrenocortical adenomas or carcinomas.[145–147] Common clinical and laboratory problems in these cats included hypokalemia, associated muscle weakness, metabolic alkalosis, and arterial hypertension. Each cat had an increased plasma [aldosterone]: > 5000 pmol/L with a RI of 540–1080 pmol/L,[145] 877–14,653 pmol/L with a RI of 150–430 pmol/L, > 5000 pmol/L with a RI of 87–224 pmol/L, or 1253 to > 3329 ng/dL with a RI of 194–388 ng/dL.[148]
   2. Adrenocortical carcinomas in some cats with hyperaldosteronism produce excessive amounts of progesterone in addition to aldosterone.[149] Diabetes mellitus is common in

Table 18.5. Aldosterone concentrations from ACTH stimulation tests in dogs

Clinical state	Aldosterone concentration (pg/mL)						
	Study 1 (mean ± 2 SD)[143]			Study 2 (range of observed values)[144]			
	n	Before ACTH	1 h after ACTH[a]	*n*	Before ACTH	1 h after ACTH[b]	2 h after ACTH
Healthy	7	203 ± 142	397 ± 188	21	5–345	91–634	71–758
Hypoadrenocorticism	5	167 ± 30	162 ± 56	6	0–56	—	0–36
PDH	28	124 ± 120	331 ± 216	—	—	—	—
Iatrogenic hypercorticism[c]	9	126 ± 118	213 ± 132	—	—	—	—
NAI[d]	—	—	—	5	> 1200	> 1200	—
Diabetes mellitus + mitral insufficiency	—	—	—	1	10	—	20

[a] 0.5 IU/kg IV
[b] 2.2 IU/kg IM
[c] A diagnosis of iatrogenic hypercorticism was based on finding reduced cortisol concentrations in pre-ACTH and post-ACTH samples in dogs with appropriate clinical signs and history of relevant exogenous steroid treatments.
[d] Diagnoses included chylothorax (1), lymphocytic gastritis (1), glomerulonephritis and mitral insufficiency (1), acute renal failure (1), and acute renal failure plus diabetes mellitus (1). In the chylothorax and gastritis cases, the aldosterone concentrations in 2-h post-ACTH samples also were > 1200 pg/mL.

these cats, presumably related to progesterone-induced insulin resistance. It may be recognized before hypokalemia, metabolic alkalosis, hypertension, and hyperaldosteronemia. Excessive production of other corticosteroids (e.g., corticosterone) can occur; the adrenal response to exogenous ACTH stimulation may be suppressed, and adrenal insufficiency may occur after adrenalectomy.

3. Idiopathic hyperaldosteronism with bilateral hyperplasia of the zona glomerulosa has also been described in cats.[150] The main findings in these cats were hypokalemia, weakness, and hypertension, but plasma aldosterone concentrations were only mildly increased or, more often, in the upper half of the RI, thus greater than expected with hypokalemia. Plasma renin activity was decreased or near the lower reference limit, and the ratio of [aldosterone] to renin activity was increased (see Section IV below). Idiopathic adrenal hyperplasia has also been suspected in a dog.[151]
4. Confirmatory testing is based on the expectation of autonomous aldosterone secretion.[152]
   a. Increased aldosterone activity results in Na^+ retention that causes subsequent H_2O retention and an expanding ECF volume. This causes negative feedback on production and release of renin. Plasma renin activity is expected to be decreased or, in the case of idiopathic hyperplasia, near the lower reference limit. Plasma aldosterone-to-renin ratios are increased because of the combination of higher aldosterone concentrations and lower renin activity (see Section IV below).[150]

(1) Very few laboratories offer measurement of plasma renin activity, and RIs vary. Note: It is renin (pronounced "ree nin"), not the gastric protease rennin (pronounced "renn in").
(2) Plasma renin is very unstable; samples require special collection, handling, and storage conditions.

b. Fludrocortisone suppression test and urinary aldosterone-to-creatinine ratio[153]
(1) Fludrocortisone is a mineralocorticoid. In health, it suppresses the release of aldosterone from adrenal glands, whereas it poorly suppresses or does not suppress aldosterone from aldosterone-producing neoplasms or adrenal glands with idiopathic cortical hyperplasia.
(2) Fludrocortisone is administered orally for 4 d (0.05 mg/kg every 12 h), and urine is collected for aldosterone and creatinine assays. In clinically healthy cats, the urinary aldosterone-to-creatinine ratio was suppressed > 40 %, but it was not suppressed in most cats with primary hyperaldosteronism.

c. Fludrocortisone suppression test and serum [aldosterone][154]
(1) The basic principle is the same as that of the preceding procedure, but less fludrocortisone is administered over fewer days, and serum – not urine – [aldosterone] is measured.
(2) Fludrocortisone is administered orally (0.05 mg/kg every 12 h) for a total of three doses; serum aldosterone concentrations before and after fludrocortisone administration are compared. In clinically healthy cats, the serum [aldosterone] decreased to < 40 % of the baseline [aldosterone]. The study did not include cats with primary hyperaldosteronism.
(3) Serum [cortisol] was also suppressed by fludrocortisone.

III. Aldosterone concentrations in canine disorders other than primary hyperaldosteronism

A. Hypoadrenocorticism: There is not a clear separation of pre-ACTH aldosterone concentrations between healthy dogs and dogs with hypoadrenocorticism. However, aldosterone concentrations in post-ACTH samples were lower in dogs with hypoadrenocorticism than in clinically healthy dogs (Table 18.5).[63,143]

B. HAC: In one study, the pre-ACTH [aldosterone] was lower in 28 dogs with PDH than in healthy dogs, but there was not a significant difference between groups for the post-ACTH [aldosterone] (Table 18.5).[143] Similarly, in a study of 31 dogs with PDH, 5 dogs with FAN, and 12 healthy dogs, unstimulated aldosterone concentrations were significantly lower in the PDH dogs than in the healthy dogs.[155] These authors suggested that under chronic ACTH stimulation, some of the aldosterone-secreting cells converted to cortisol-secreting cells as has been described in experimental sheep and rat studies. The [aldosterone] was significantly greater in the five dogs with FAN than in the dogs with PDH, probably related to increased renin (angiotensinogenase) concentrations from changes in blood volume and electrolyte concentrations. Negative feedback on ACTH by dexamethasone decreased plasma [aldosterone] in healthy dogs but not in dogs with PDH or FAN.

C. NAI: The [aldosterone] is very high in some dogs with NAI, probably related to hypovolemia or decreased effective blood volume (Table 18.5).[144] This is the case for some dogs with whipworm (*Trichuris vulpis*) infection, sometimes called pseudo-Addison's disease.[156] Treatments such as furosemide may also affect blood volume and increase [aldosterone].

D. Mitotane or trilostane treatment: After mitotane or trilostane treatments for PDH in dogs, exogenous ACTH still stimulates secretion of aldosterone, but the response is commonly suppressed at 30 min and 60 min.[157] Serum electrolyte concentrations cannot be used to predict the post-ACTH aldosterone response.

IV. Aldosterone-to-renin ratio
   A. Plasma aldosterone concentrations are related to renin concentrations via changes in blood volume or electrolyte concentrations.
      1. Aldosterone production is stimulated by angiotensin II, which is produced by the renal release of renin in response to renal hypotension or decreased delivery of sodium to the distal tubules.
      2. Renal responses to aldosterone result in retention of Na^+ and Cl^-, which promotes plasma volume expansion and thus decreased stimulation of the renin–angiotensin system.
      3. Aldosterone concentrations are expected to be greater in people on a Na^+-restricted diet (aldosterone is needed to retain Na^+) than in people who are not on a Na^+-restricted diet. Similar findings are expected in domestic mammals.
   B. Interpretation of an increased plasma [aldosterone] should include a consideration of whether the increase is appropriate (e.g., stimulated release because of decreased effective blood volume) or inappropriate (i.e., autonomous release from an adrenal neoplasm or idiopathic hyperplasia).
      1. In a study of 22 dogs with primary hypoadrenocorticism, aldosterone-to-renin ratios were consistently lower (0.002–0.08 pmol/fmol/s = 2–80 s) than the ratios in 60 healthy dogs (0.09–1.6 pmol/fmol/s = 90–1600 s)[98] (the authors reported the ratios without units). Units of the ratios are complex because the authors measured renin activity (rather than concentration) via the generation of angiotensin I; the ratio represents the amount of aldosterone (pmol) related to renin activity (fmol of angiotensin I produced per second). Different units would yield different ratios.
      2. Increased aldosterone-to-renin ratios occur with autonomous aldosterone production in primary hyperaldosteronism; ratios would be lower if increased renin were driving production of aldosterone.
      3. Conceptually, the aldosterone-to-renin ratio should improve the interpretation of a plasma [aldosterone]. However, it probably will have limited use in clinical medicine because of sample collection requirements and availability of renin assays. As with many other hormone assays, differences in assay methods limit the usefulness of the ratio because interpretation guidelines may not be transferrable from one set of assays to another.

CONCENTRATIONS OF ADRENAL SEX AND INTERMEDIATE HORMONES

In addition to cortisol and aldosterone, some laboratories can measure serum concentrations of other corticosteroid hormones including dehydroepiandrostenedione, estradiol, androstenedione, 17OHP, progesterone, testosterone, 11-deoxycortisol, 21-deoxycortisol, and corticosterone. These sex hormones and intermediate hormones in the synthesis of cortisol, aldosterone, and sex hormones can all be produced from cholesterol in the adrenal cortex (Figure 18.8). Consequently, increased plasma

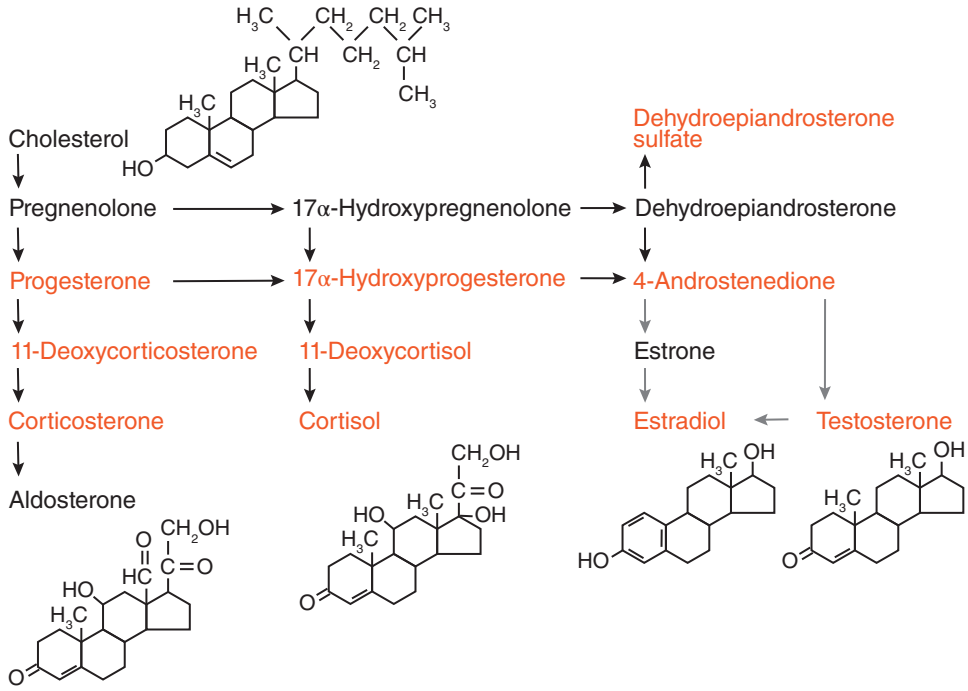


Figure 18.8. Adrenal steroidogenesis in hyperadrenocorticism. Most diagnostic efforts for HAC are directed toward documenting inappropriate production of cortisol. However, increased concentrations of other corticosteroids (*orange font*) have been reported in cases of canine HAC (see text), as have concentrations of some of their metabolites (e.g., 21-deoxycortisol from 17OHP). The major synthetic relationships of these steroids are illustrated; not all pathways are shown, and there may be species differences. Each arrow represents an enzymatic conversion. Molecular structures show the similarities of cholesterol, aldosterone, cortisol, estradiol, and testosterone. Adrenal synthesis of estradiol and testosterone are miniscule in health (*grey arrows*), but an adrenal neoplasm with altered synthetic pathways could secrete more. Corticosteroids secreted from adrenal glands may be further metabolized to certain measured hormones (e.g., estradiol) in other tissues.

concentrations of these corticosteroids before or after exogenous ACTH stimulation may reflect adrenal dysfunction.

I. Dogs with HAC and supportive laboratory test results: Many dogs with HAC have increased pre- or post-ACTH concentrations of one or more intermediate steroids in the cortisol synthesis pathways, e.g., 17OHP, but the role of these intermediates in disease is unclear. Post-ACTH increases in concentrations of these hormones also commonly occur with NAI.
   A. In a study of 53 dogs with confirmed PDH or FAN, 69 % had an exaggerated increase in [17OHP] with the ACTH stimulation test, and 79 % had an exaggerated increase in [cortisol].[158]
   B. Similarly, ACTH stimulation caused exaggerated increases in cortisol, progesterone, and 17OHP concentrations in 84 %, 88 %, and 91 %, respectively, of 32 dogs with HAC, but it

should be noted that this was based on comparison with the central 95 % of noncortisol results for just six healthy dogs.[122] The post-ACTH [progesterone] was increased in all five dogs for which the post-ACTH [cortisol] was not increased, and the [17OHP] was increased in four of these five dogs. However, ACTH stimulation also caused exaggerated increases in cortisol, progesterone, and 17OHP concentrations in 34 %, 45 %, and 41 %, respectively, of 29 dogs with NAI, and of the 10 dogs with NAI for which the post-ACTH [cortisol] was not increased, the [progesterone] was increased in 5 dogs and the [17OHP] was increased in 6 dogs.

C. Another study included 127 dogs with suspected HAC. After ACTH stimulation, 59 (46 %) had an exaggerated increase in [cortisol]. Of those 59 dogs, 42 (71 %) had an exaggerated increase in [17OHP].[159] However, an exaggerated increase in [17OHP] was also found in 31 % of dogs that had neoplasms not related to pituitary or adrenal glands, supporting that adrenocortical hyperplasia occurs secondarily to NAI.

D. Statistically, baseline and post-ACTH 17OHP concentrations were greater in 46 dogs with HAC than in 46 dogs without HAC (29 healthy dogs, 17 dogs with NAI). However, routine measurement of [17OHP] for diagnosing HAC concentrations was not recommended because of considerable overlap in concentrations between groups.[160]

E. In a study including 9 dogs with FAN and 11 dogs with PDH, most dogs had exaggerated increases in at least one sex hormone after ACTH stimulation, with increases being greatest in the dogs with adrenocortical carcinomas.[161] However, the responses were variable and did not appear useful for differentiating PDH from FAN.

F. In a study of 15 dogs with PDH, 8 dogs with NAI, and 19 healthy dogs, post-ACTH plasma concentrations of cortisol, 17α-hydroxypregnenolone, 17OHP, and 21-deoxycortisol were greater in PDH dogs than in healthy dogs.[162] Post-ACTH plasma concentrations of cortisol, 17OHP, and 11-deoxycortisol were greater in dogs with NAI than in healthy dogs; therefore, ACTH-induced increases in noncortisol corticosteroid concentrations are not specific for HAC. Overall, the post-ACTH cortisol concentrations had the best diagnostic value for HAC of all the steroid concentrations measured in this study.

II. Dogs with clinical signs of HAC but lacking laboratory support based on dexamethasone suppression testing, ACTH-stimulated [cortisol], and UCCR (i.e., so-called atypical, occult, or suspected HAC)

A. Increased pre- or post-ACTH concentrations of various sex hormones have been reported in such dogs with adrenal carcinomas, but it is not known (i) if these hormones contribute to either clinical signs or the suppression of ACTH secretion, (ii) if cortisol is responsible despite test results, or (iii) if measurement of adrenal sex and intermediate hormone concentrations has diagnostic value for adrenal neoplasms given similar increases in some dogs with NAI and the ability to detect adrenal masses by imaging.

B. In a study of 24 dogs with clinical features of HAC, 3 dogs with adrenal masses maintained low cortisol concentrations and lacked abnormal responses to exogenous ACTH and dexamethasone but had exaggerated post-ACTH increases in [17OHP] and undetectable concentrations of endogenous ACTH.[163] Similarly, each of the other 10 dogs without LDDST or ACTH stimulation results to support HAC (and the 11 dogs with such support) had an exaggerated post-ACTH increase in [17OHP], and clinical response of some dogs to adrenalectomy, mitotane, or trilostane supported diagnoses of HAC. The exaggerated 17OHP responses to ACTH also resolved in some dogs after treatment.

C. Two neutered dogs with adrenocortical carcinomas and clinical signs of HAC had suppressed cortisol responses to exogenous ACTH, decreased endogenous ACTH concentrations, and increases of various other adrenal hormone concentrations, either at baseline or after ACTH stimulation.[164]
   1. One dog initially had an increased post-ACTH [estradiol] and increased pre- and post-ACTH concentrations of progesterone and 17OHP. After 2 wk of mitotane treatment, pre-ACTH concentrations of progesterone and 17OHP had increased, and pre-ACTH concentrations of testosterone and dihydroepiandrostenedione were increased compared to reference values (pre-mitotane results were not reported).
   2. The other dog had increased pre- and post-ACTH concentrations of androstenedione, estradiol, progesterone, and 17OHP, as well as an increased post-ACTH concentration of testosterone. These increases all corrected to WRI after adrenalectomy, as did the cortisol response to ACTH.

D. In a study that included eight dogs with adrenal neoplasms and signs of HAC, one of the three dogs with suppressed cortisol responses to ACTH had an increased post-ACTH [17OHP], as did one dog without a suppressed ACTH response.[116] No other hormones were assessed.

III. Scottish terriers with hyperphosphatasemia[165,166]

A. Affected dogs typically appear healthy but have persistent and progressive increases in serum alkaline phosphatase (ALP) activity, primarily of the corticosteroid-induced isoform. They also have increased UCCRs compared to healthy dogs and develop vacuolar hepatopathy. Although the typical clinical signs for HAC are lacking, these laboratory findings can suggest HAC, so corticosteroid responses to ACTH stimulation have been assessed.

B. Some of these hyperphosphatasemic dogs have an increase in basal [cortisol] and an exaggerated cortisol response to exogenous ACTH. Many also have increased concentrations of various other corticosteroids (androstenedione, estradiol, progesterone, and 17OHP) pre-or post-ACTH stimulation, but the predominant hormone and pattern have been inconsistent. Fewer of the Scottish terriers without hyperphosphatasemia have had increased concentrations of cortisol, androstenedione, and estradiol pre- and post-ACTH, but similar proportions have had exaggerated increases in progesterone and 17OHP concentrations.[165] Of note, however, is that increased concentrations of several of these hormones were reported in more of the comparative healthy dogs than would be expected for appropriately representative RIs.[166]

C. Because dexamethasone administration decreased concentrations of cortisol, androstenedione, progesterone, and 17OHP in affected dogs, feedback pathways appear intact.[166] A potential cause of the disorder is a genetic defect causing decreased enzymatic degradation of corticosteroid hormones, but the cause is currently unknown.

IV. Cats with adrenal neoplasms

A. Increased plasma [progesterone] in cats with hyperaldosteronism is discussed above in the section on primary hyperaldosteronism.

B. Hyperprogesteronemia with a prominent exaggerated response to exogenous ACTH was reported in a castrated male cat with an adrenocortical carcinoma and clinical signs of bilaterally symmetrical alopecia, polyuria, polydipsia, and recent aggressive behavior.[167] This cat had negative dexamethasone suppression and ACTH stimulation tests for HAC,

including a low post-ACTH [cortisol]. However, clinical signs and hyperprogesteronemia resolved after adrenalectomy.

C. Rarely, cats have functional adrenal adenomas or carcinomas that cause behavioral or physical changes associated with increased serum concentrations of estradiol or testosterone but without clinical or laboratory evidence of hypercortisolism or hyperaldosteronsim.[168,169] Because of limited hormonal testing in most cases, the degree to which various other sex and intermediate corticosteroids were secreted is unknown. Clinical and laboratory abnormalities related to excesses of these sex hormones have resolved with adrenalectomy.

THORN TEST AND MODIFIED THORN TEST

I. Thorn test[170] (included for historical and conceptual perspective)
   A. Instead of directly measuring [cortisol] after an ACTH injection for diagnosing hypoadrenocorticism, changes in [cortisol] can be indirectly and roughly assessed via changes in blood [eosinophil] (Thorn test). An [eosinophil] is measured directly, not calculated from the WBC differential count and eosinophil percentage.
   B. In a healthy dog, blood [eosinophil] is expected to decrease if blood [cortisol] increases. Failure to see a decrease in [eosinophil] after administration of ACTH suggests the presence of hypoplastic or atrophied adrenal glands.
   C. Increased ease of obtaining serum or plasma cortisol concentrations and their superior diagnostic value have made the Thorn test nearly obsolete. However, the relationship between blood [cortisol] and blood WBC concentrations should be remembered when interpreting routine leukogram results.

II. Modified Thorn test[171] (included for historical and conceptual perspective)
   A. In this assessment, a neutrophil-to-lymphocyte ratio is calculated from WBC differential counts determined on blood films before and 4 h after intravenous administration of ACTH.
   B. Based on data from 13 dogs, a 30 % increase in the neutrophil to lymphocyte ratio after ACTH was considered an appropriate response to the release of cortisol. Concurrently, the blood [eosinophil] (measured directly and not calculated from the differential count) decreased by at least 50 % after ACTH. The authors recommended that evaluation of blood WBC changes could be used as a screening method for hypoadrenocorticism; if the expected changes were not seen, then plasma samples could be submitted for measurement of cortisol concentrations.

CATECHOLAMINES (EPINEPHRINE, NOREPINEPHRINE) AND THEIR METABOLITES (METANEPHRINE, NORMETANEPHRINE)

I. The major diagnostic reason for measuring concentrations of catecholamines or their metabolites is to pursue diagnosis of a pheochromocytoma or a rare functional paraganglioma (neoplasm of paraganglion chromaffin cells outside the adrenal gland).
   A. Although the major clinical features (e.g., hypertension, tachycardia, weakness, polyuria, polydipsia) are associated with effects of epinephrine and norepinephrine, plasma metanephrine or normetanephrine concentrations or quantitative urinary excretion of metanephrine or normetanephrine have better diagnostic sensitivity and specificity than the

assessment of epinephrine or norepinephrine in plasma or urine.[172–174] Adrenal chromaffin cells are the major source of metanephrine and normetanephrine, which they continually form by catecholamine methylation, independent of episodic catecholamine secretion.[13]

B. In human medicine, diagnostic accuracy for pheochromocytomas and paragangliomas appears best with assessment of plasma or urine free rather than total (deconjugated) normetanephrine and metanephrine concentrations, consistent with the fact that much of the conjugated metanephrine and normetanephrine reflects nonadrenal catecholamine production.[37] LC-MS/MS is considered the method of choice.

II. Increased plasma concentrations of normetanephrine and metanephrine

A. When LC-MS/MS results for11 dogs with pheochromocytomas were compared to those for 6 dogs with NAI but similar clinical presentations, for 25 dogs with HAC, and for 80 healthy dogs, plasma free normetanephrine concentrations had high diagnostic accuracy for pheochromocytoma, overlapping slightly with just 2 HAC dogs that had mild increases.[36] In contrast, free metanephrine concentrations in the pheochromocytoma group overlapped more with those of the HAC group and even more so with those of the NAI group, yielding only moderate diagnostic accuracy for pheochromocytomas.

B. When LC-MS/MS results for 8 dogs with pheochromocytomas were compared to those for 11 dogs with adrenocortical neoplasms, 15 dogs with NAI, and 11 healthy dogs, plasma free normetanephrine concentrations were greater for dogs with pheochromocytomas than for the dogs in each of the other groups, and with little overlap among groups.[175] Plasma free metanephrine concentrations were significantly greater for dogs with pheochromocytomas compared to healthy dogs and dogs with HAC, but not compared to dogs with NAI.

C. When LC-MS/MS results for a cat with a pheochromocytoma were compared to those for 10 healthy cats, the plasma [free normetanephrine] was greater than for all healthy cats, but the plasma [metanephrine] was not clearly increased.[26] Similarly, using a high-pressure liquid chromatography method, the plasma [free normetanephrine] for a different cat suspected of having a pheochromocytoma was greater than that of all other cats tested (seven healthy and five with NAI), whereas the [free metanephrine] was not clearly increased compared to either group.[176]

III. Increased urinary excretion of normetanephrine and metanephrine

A. The urinary excretion of total normetanephrine (expressed as urinary normetanephrine-to-creatinine ratio) for 7 dogs with pheochromocytomas was greater than for all 10 dogs with hypercortisolism, all 14 dogs with NAI, and all 10 healthy dogs.[172] Urine samples were acidified to a pH $\leq$ 2 and analyzed by high-performance liquid chromatography with amperometric detection. One dog in the pheochromocytoma group had plasma concentrations of total normetanephrine and free normetanephrine that overlapped with concentrations in dogs with NAI or good health; plasma concentrations were measured by high-performance liquid chromatography with coulometric detection. As in other studies, normetanephrine appeared to be superior to metanephrine in identifying dogs with pheochromocytomas.

B. When results from 7 other dogs with pheochromocytomas were compared to data from 10 healthy dogs, the normetanephrine-to-creatinine ratios of the dogs with pheochromocytomas were consistently greater than those of the healthy dogs, and they had more diagnostic utility than the ratio for urinary excretion of metanephrine.[173] Similarly, when results from still another 7 dogs with pheochromocytoma were compared with those of 14 dogs with HAC and 10 healthy dogs, the normetanephrine-to-creatinine ratios of

dogs with pheochromocytomas were consistently greater than those of the healthy dogs and dogs with HAC.[174] The urinary ratios for metanephrine were similar for dogs with pheochromocytomas and HAC.

C. When LC-MS/MS results for a cat with a pheochromocytoma were compared to those for 10 healthy cats, the urine normetanephrine-to-creatinine ratio was greater than that for each healthy cat, and the urine metanephrine-to-creatinine ratio was greater than the ratio for 9 of the 10 healthy cats.[26]

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 19

PERITONEAL, PLEURAL, AND PERICARDIAL EFFUSIONS

Michael A. Scott and Steven L. Stockham

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Table 19.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
ALP	Alkaline phosphatase
Chol:TG	Cholesterol to triglyceride
Crt	Creatinine
EDTA	Ethylenediaminetetraacetic acid
FIP	Feline infectious peritonitis
Hct	Hematocrit
LD	Lactate dehydrogenase
NT-proBNP	Amino-terminal proB-type natriuretic peptide
RBC	Red blood cell (erythrocyte)
SG	Specific gravity
TG	Triglyceride
TG:Chol	Triglyceride to cholesterol
TNCC	Total nucleated cell concentration
TP	Total protein
TP_{ref}	Total protein determined by refractometry
TS_{ref}	Total solids determined by refractometry
WBC	White blood cell (leukocyte)

GENERAL CONCEPTS AND DEFINITIONS

I. Effusions accumulate in body cavities because of physiologic or pathologic processes. Results from the analysis of the effusion and other clinical information are used to determine which process or processes are present in a given animal. This chapter provides an introduction to the results of cavitary fluid analyses and how the results relate to pathologic processes. Most of the chapter is directed toward pleural and peritoneal effusions; the last section contains information about pericardial fluid. Pleural and peritoneal effusions are often referred to as thoracic and abdominal effusions (respectively), but thoracic or abdominal fluid can be located in areas other than the pleural or peritoneal cavities. Several excellent books can be consulted for more information about analytical methods and the microscopic features of cells and other structures in effusions.[1–6]

II. The pleural and peritoneal cavities are lined by a layer of mesothelial cells (mesothelium) with two surfaces: the visceral surface that covers viscera (lungs, intestine, etc.) and the parietal surface that covers mediastinum and the pleural and peritoneal walls. In most species, a small amount of clear serous fluid in the cavities provides a lubricant and medium for transport of electrolytes and other substances. The cavities are called *serous* because, in health, they contain a clear, watery fluid that grossly resembles serum. Pleural and peritoneal fluids are formed by similar processes that involve the forces of Starling's law and anatomic structures. Generally, a plasma filtrate leaves capillaries, enters interstitial space, and diffuses into a serous cavity, from which it is removed by the lymphatic system and returned to plasma. *Pleural fluid* is formed in health by diffusion of fluid through the visceral mesothelium to the pleural cavity (Figure 19.1).[7] The fluid has a low [protein] and low cellularity. *Peritoneal fluid* is formed similarly, with most of the fluid removed by diaphragmatic lymphatic vessels.[8]

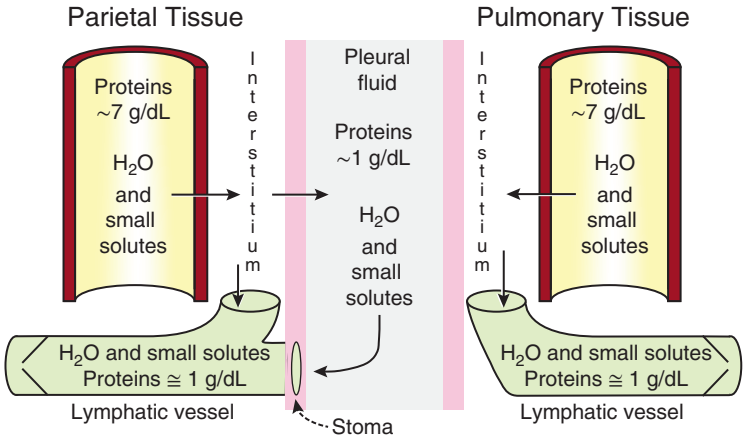


Figure 19.1. Pleural fluid formation and removal. Forces of Starling's law of capillaries move solute-rich, protein-poor fluid (H_2O containing small solutes such as glucose, urea, and electrolytes) from capillaries to the parietal and pulmonary interstitial spaces. The parietal capillary beds and parietal pleura are relatively impermeable to proteins, so the fluid that diffuses from the interstitium to the pleural space has a lower [TP] (~1–2 g/dL). In health, pleural fluid is drained by the parietal lymphatic vessels. The drainage is powered by the lymphatic pump that creates a negative pressure to pull fluid from the interstitium. Evidence indicates that the visceral pleural barrier is impermeable to H_2O and solutes in most mammals during health.

III. Composition of pleural fluid and peritoneal fluid in health.

A. The chemical composition of a body cavity fluid is primarily determined by permeability of capillaries to H_2O and solutes and, to a lesser extent, permeability of pleural and peritoneal mesothelium.

1. Capillaries are permeable to H_2O, electrolytes (e.g., Na^+, K^+, Cl^-, Ca^{2+}, bicarbonate, and phosphates) and small nonprotein solutes (e.g., glucose, urea, and Crt). Other than [protein], the interstitial fluid adjacent to most capillaries has electrolyte, urea, glucose, and Crt concentrations similar to plasma.
2. Interstitial fluid is the source of most pleural and peritoneal fluid proteins. Tissue variations in capillary permeability to plasma proteins cause variations in interstitial [TP]. In people, the interstitial fluid [TP] is near 1.5 g/dL in skeletal muscle, near 2.0 g/dL in subcutaneous tissue, near 4 g/dL in intestine, and near 6 g/dL in liver.[9] The [TP] of pulmonary and pleural interstitium was not found, but the [TP] of pleural fluid in health is near 1–2 g/dL.[10]

B. Pleural and peritoneal fluids in healthy animals are not expected to contain RBCs and should have low concentrations of nucleated cells (e.g., mesothelial cells, lymphocytes, macrophages, and neutrophils). However, thoracocentesis or abdominocentesis typically damages small blood vessels, and thus a few contaminating RBCs and rare blood WBCs are not unusual in collected fluids.

C. Canine pleural and peritoneal fluids

1. The amount of pleural and peritoneal fluids in healthy dogs is very small, so it is difficult to collect sufficient fluid for analysis. In one study, a mean of 1.4 mL was aspirated postmortem from the pleural space of 14 healthy dogs weighing 12–16 kg.[11] In another study, a mean of 2.35 mL was the total estimated pleural fluid volume of six healthy dogs weighing 6.5–12.5 kg; the value was the sum of a mean 0.55 mL that was physically collected (postmortem) plus a calculated mean of 1.8 mL forming the

fluid film lining the pleural wall.[12] The volume of peritoneal fluid in healthy dogs may be similar or a little greater.[13]

2. There is very little information about the expected composition of these fluids in health, but data from multiple sources suggest they should have these features: colorless to slightly yellow, clear, a [TP_{ref}] < 2.5 g/dL, a TNCC < 3.0 × 10^3/μL (mode < 1.0 × 10^3/μL), and a mixture of mostly mesothelial cells and macrophages with few neutrophils and lymphocytes.[13] The "[TP_{ref}] < 2.5 g/dL" feature is based on use of refractometers with scales not extending below 2.5 g/dL. The actual [TP] is most likely 0.9–2.0 g/dL and was 0.9–1.4 g/dL in the pleural fluid of four healthy dogs when measured by the biuret method;[12] electrophoresis of the same four samples showed that 51–69 % of the protein was albumin.
3. Typically, canine cavitary fluid is collected when there is an effusion, and the task is determining the cause of the effusion, not whether the fluid is abnormal.

D. Feline pleural and peritoneal fluids: As in dogs, the amounts of pleural and peritoneal fluids in healthy cats are very small, so it is difficult to collect sufficient fluid for analysis. If fluid can be collected, the fluid probably represents an effusion.

E. Equine pleural and peritoneal fluids: Healthy horses do have enough cavitary fluid for collection and analysis, but the reported findings vary considerably (Table 19.2). The [TP_{ref}] and TNCC values for "healthy" horses seemed to decrease after more effective anthelmintic drugs reduced the incidence of subclinical parasite infestations, but

Table 19.2. Reported features of pleural and peritoneal fluids of healthy horses[a]

	Pleural[14]	Peritoneal[15]	Peritoneal[16]	Peritoneal[17]
Number of horses	18	25	20	20
Volume collected (mL)	2–8	10–100	10–100	—
Color	Yellow or red-yellow	Light yellow	Light yellow	—
Clarity	Clear to hazy	Clear to sl. turbid	Clear to sl. turbid	—
TP_{ref} (g/dL)	< 4.2[b]	—[c]	0.1–2.5[d]	0.2–1.5
TNCC (× 10^3/μL)	0.8–12.1	0.2–9.0	0.0–4.6	1.5–10.1
Neutrophils	0.4–10.3 × 10^3/μL	36–78 %	80–98 %	22–82 %
Lymphocytes	0.0–0.7 × 10^3/μL	0–29 %	1–11 %	1–19 %
Monocytes/macrophages	0.0–2.6 × 10^3/μL	3–50 %	1–17 %	19–68 %
Mesothelial cells	—	—	Occasional	—
Eosinophils	0.0–0.2 × 10^3/μL	0–3 %	0–7 %	0–5 %
Mast cells	—	—	Occasional	—
RBCs (× 10^3/μL)	22–540	—	0.2–5.4	—

[a] Although clinically healthy, the [TP_{ref}] > 2.0 g/dL and neutrophil data suggest subclinical exudation in some of the horses.
[b] Fifteen of 18 samples had [TP_{ref}] ≤ 2.5 g/dL.
[c] [TP] data were not included in the table as there were inconsistencies in the reported data.
[d] Eighteen of 20 samples had [TP_{ref}] ≤ 1.7 g/dL.
sl., slightly

Table 19.3. Reported features of peritoneal fluids of healthy cattle[a]

	From[b] Wilson et al.[18]	From Anderson et al.[19]
Number of cattle	19	8
TP_{ref} (g/dL)	0.1–4.6	2.2–4.0
$Fibrinogen_{ref}$ (g/dL)	0.1–0.4	—
TNCC ($\times 10^3/\mu L$)	5.0–30.0	0.4–3.0
Neutrophils	12–58 %	$< 2.2 \times 10^3/\mu L$
Lymphocytes	1–28 %	$< 0.2 \times 10^3/\mu L$
Monocytes/macrophages	1–28 %	$< 1.0 \times 10^3/\mu L$
Eosinophils	25–72 %	$< 0.6 \times 10^3/\mu L$

[a] Although clinically healthy, the [TP_{ref}] > 2.0 g/dL and neutrophil component suggest subclinical exudation in some of the cattle.
[b] Most data extracted from graphs

contemporary reference values are scarce, particularly from properly designed studies published in peer-reviewed journals. The published data summarized in Table 19.2 indicate that the TNCC in pleural or peritoneal fluid of horses can be at least as high as 10,000/μL in apparently healthy horses, but it is less than 5000/μL in many healthy horses, so a value of 10,000/μL could reflect inflammation. For pleural and peritoneal fluids, neutrophil percentages need to be interpreted with knowledge of the fluid's TNCC and the quantity of fluid that is in the cavity.

F. Healthy cattle may have enough peritoneal fluid for collection and analysis. However, pathologic effusions are uncommon, and analysis of effusions is even less common. Reported features of peritoneal fluid in healthy cattle are listed in Table 19.3, but some of the values are much greater than what is expected in healthy animals of most species. Thus, the findings may not truly represent data from healthy cattle. TNCC may be increased during the first 2 wk postpartum.[20]

G. Healthy llamas and alpacas have a small quantity of peritoneal fluid that can be collected with a teat cannula. Of 22 healthy llamas and alpaca, nearly all had [TP_{ref}] ≤ 2.5 g/dL (12 had [TP_{ref}] ≤ 1.0 g/dL), TNCC < $3.0 \times 10^3/\mu L$, and a majority of the nucleated cells were neutrophils.[21] A few animals had unexplained higher [TP_{ref}] and/or TNCC. Small amounts (< 1 mL) of pleural fluid have been collected from healthy llamas. Most fluids had [TP_{ref}] ≤ 2.7 g/dL, TNCC < $1.5 \times 10^3/\mu L$, and most nucleated cells (80–100 %) were small lymphocytes.[22]

IV. Definitions

A. *Cavitary* is the adjective for a *cavity*, a gas or fluid-filled space within a relatively solid object, e.g., the body or an organ. Peritoneal, pleural, and pericardial spaces are referred to as body cavities; fluids within them are cavitary effusions.

B. *Effusion* is (i) the process by which fluid accumulates in a body space or cavity, and (ii) the fluid that has accumulated in a body space or cavity.

C. *Ascites* is the fluid accumulated in the peritoneal cavity. It can be a transudate, exudate, or other type of effusion (e.g., hemorrhagic ascites).

D. *Transudation* is the passage of fluid or solute through a membrane because of imbalances in hydraulic or oncotic pressure gradients.

E. A *transudate* is an effusion produced by imbalances in oncotic or hydraulic pressure within capillary beds. Imbalances influence the loss or resorption of fluid. Protein concentration varies with the site of formation.

F. *Hydrostatic pressure* is the energy (pressure) of a fluid at rest (used by many in reference to hydraulic pressure); the pressure is created by gravity.
G. *Hydraulic pressure* is the energy (pressure) of a fluid in motion; usually the motion is due to the actions of a pump (e.g., heart).
H. *Exudation*, in the context of cavitary effusions, is an exuding, oozing, or discharge of fluid, solids, or cells from vessels to body cavities.
I. An *exudate* is an effusion produced by increased vascular permeability to plasma proteins because of inflammation; the cellular content of an exudate varies with the type and duration of the inflammation. *Pyothorax* refers to a pleural exudate that is cellular enough to be turbid and pus-like, sometimes blood-tinged; it is usually caused by a bacterial infection. *Pyoabdomen* is a less common term that may be used in reference to similar peritoneal effusions.
J. *Hemorrhage* is the escape or loss of blood from blood vessels or heart.
K. *Lymphorrhage* is the escape or loss of lymph from lymph vessels.
L. *Serosanguineous* (SEER oh san GWIN ee us) is a gross descriptive term for pink fluid with features of serous (serum-like) fluid (*sero*) containing a relatively small amount of blood (*sanguineous*, from the Latin *sanguineus* meaning "from blood"). The term *serosanguinous* is often used in its place.
M. Based on its use by various publications, *modified transudate* does not have a single definition. This classification is not recommended by the authors.
    1. Pathologic processes that produce these effusions are varied and not consistently described. The origin of the *modified transudate* category of effusion is unknown. It is rarely described in the human literature and was not present in a 1967 veterinary clinical pathology textbook.[23] Benjamin wrote in 1961 that "a typical transudate can usually be readily distinguished from a typical exudate; but many extravascular fluids are modified transudates or modified exudates with some characteristics of both, so that recognition of the etiologic process may be difficult without a more complete examination of the fluid."[24] Modified transudate was listed as one type of effusion in a 1977 veterinary clinical pathology textbook and was defined as "an accumulation of transudative type fluid which has been modified by the addition of proteins and/or cells."[25] It is now a common label or classification even though it may not reflect the pathologic process responsible for the effusion.
    2. The modified transudate classification is usually based on laboratory data rather than the pathologic process or processes that produced the effusion. Basically, this classification has been used for effusions with a [TP_{ref}] and/or TNCC in a grey zone between protein-poor transudates and typical exudates.
    3. A variety of effusions have been considered to be modified transudates, including hemorrhagic, chylous, congestive, neoplastic, and low-grade inflammatory effusions. Increased hydraulic pressures contribute to transudation in congestive heart failure, but transudation is not the primary mechanism responsible for the fluid that accumulates in hemorrhagic, most chylous, and most neoplastic effusions. In this chapter, effusions are not classified as modified transudates, because the classification does not clearly communicate the pathologic processes that led to the effusions, and many of the so-called *modified transudates* do not accumulate because of transudation and subsequent modification.

V. Basic concepts of Starling's law of capillaries (Eq. 19.1) are needed to understand the pathogeneses of cavitary effusions. In the context of most capillary beds, the major variables are illustrated in Figure 19.2.

$$\text{Net filtration} = \text{LpS}\,[\Delta \text{ hydraulic pressure} - \Delta \text{ oncotic pressure}]$$
$$= \text{LpS}\,\left[\left(P_{cap} - P_{if}\right) - s\left(\pi_{cap} - \pi_{if}\right)\right] \quad (19.1)$$

Net filtration = net flux of fluid from capillary to interstitium

Lp = unit permeability (porosity) of the capillary

S = surface area available for fluid movement

s = reflection coefficient of proteins across the capillary wall

P_{cap} = hydraulic pressure of plasma

P_{if} = hydraulic pressure of interstitial fluid

π_{cap} = oncotic pressure of plasma in capillary

π_{if} = oncotic pressure of interstitial fluid

A. A majority of the fluid that enters the interstitium from plasma returns to the plasma via the permeable venous capillaries (capillaries that open into venules). The remainder enters the lymphatic vessels and returns to the blood via the thoracic duct (most of the body) or the right lymphatic duct (parts of head, neck, and thorax).

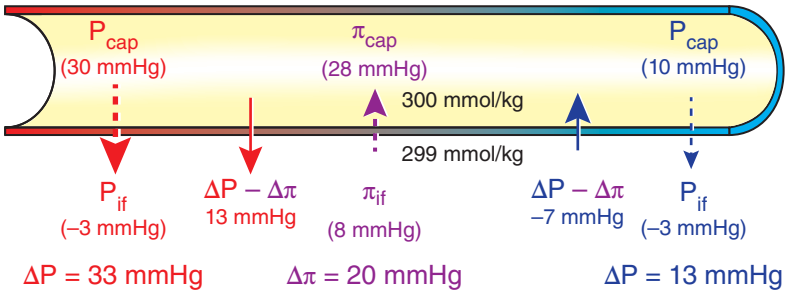


Figure 19.2. Starling's forces in capillary beds. The movement of fluid out of and into capillaries as the blood flows through common capillary beds is dependent on pressure gradients created by Starling's forces. As common capillaries are nearly impermeable to plasma proteins, the oncotic pressure gradient ($\Delta\pi = \pi_{cap} - \pi_{if}$) remains nearly constant as blood flows from arterioles to venules; in this example, $\Delta\pi$ is 20 mmHg.

- On the arterial side of the capillary bed (*left*), the hydraulic pressure in the vessel (P_{cap}) is greater than the hydraulic pressure in the interstitial fluid (P_{if}) and thus there is a hydraulic pressure gradient ($\Delta P = P_{cap} - P_{if}$); in this example, the ΔP is 33 mmHg. Because $\Delta P > \Delta\pi$, the positive difference ($\Delta P - \Delta\pi$ = 13 mmHg in example) forces the movement of solute-rich, protein-poor fluid into the extravascular space. This fluid contains nutrients for tissues.
- On the venous side of the capillary bed (*right*), the P_{cap} is much less than it was in the arterial side and there is a negative P_{if} created by the lymphatic pump. These two factors create a relatively small ΔP (13 mmHg in this example) which is less than the $\Delta\pi$. Because the $\Delta\pi > \Delta P$, the negative difference ($\Delta P - \Delta\pi$ = –7 mmHg in example) forces the movement of solute-rich, protein-poor fluid from the extravascular space into the blood. This fluid contains metabolic waste.
- Over the entire capillary bed, there is a net flow of fluid from vessels to interstitial space. Fluid does not accumulate in health because of adequate lymphatic drainage that carries fluid and solutes from interstitial fluid to blood.

Note that the osmolality in the blood is only slightly higher than the osmolality in the interstitial fluid because proteins contribute minimally (~1 mmol/kg) to osmolality compared to electrolytes and nonprotein solutes (~300 mmol/kg). Despite this minimal contribution to osmolality, proteins directly and indirectly (see Gibbs–Donnan effect, Chapter 7) create the colloid osmotic gradient (also called oncotic gradient or $\Delta\pi$).

B. Most capillary walls have minimal permeability to plasma proteins, but some proteins do enter the interstitium and the resultant lymph. The proteins in the interstitial fluid create an extravascular oncotic pressure (colloid osmotic pressure) (see Chapter 7, Colloid Osmotic Pressure).
   1. Oncotic pressure in the interstitium of skeletal muscle is about 30 % of the plasma oncotic pressure in health.[26]
   2. Oncotic pressure in the hepatic interstitium is nearly equal to plasma oncotic pressure because the walls of hepatic sinusoids are fenestrated and permeable to most plasma proteins.[26]
   3. Oncotic pressure in the pulmonary interstitium is about 70 % of plasma oncotic pressure. Alveolar capillaries are less permeable to proteins than are the hepatic sinusoids but more than are the skeletal muscle capillaries.[26]

C. The permeability of capillaries is expressed as a reflection coefficient (Eq. 19.1), which ranges from 0 (completely permeable to proteins) to 1 (impermeable to proteins). The reflection coefficient approaches 1 in most capillary beds but is smaller in hepatic and pulmonary capillary beds.

D. For most tissues, the extravascular fluid has a protein concentration much less than a plasma protein concentration, but the concentrations of other solutes (electrolytes, urea, Crt, glucose, and most small molecules) are nearly the same as those found in plasma. Minor differences in electrolyte concentrations are created by the Gibbs–Donnan effect (see Chapter 7, Colloid Osmotic Pressure, I.C.2).

E. There are "safety factors" that reduce or prevent the accumulation of extravascular fluid when there are changes in either hydraulic pressures or oncotic pressures.[26]
   1. Drainage by lymphatic vessels increases.
   2. When more protein-poor fluid enters the extravascular space because of increased hydraulic pressure, that fluid dilutes the extravascular protein concentration and thus increases the oncotic pressure gradient, promoting less fluid extravasation. Addition of extravascular fluid also increases extravascular hydraulic pressure and thus lowers the hydraulic pressure gradient.
   3. A reduction of plasma oncotic pressure (due to hypoproteinemia) may, if not too severe, be matched in time by a lowering of the extravascular protein concentration such that the oncotic pressure gradient does not change.

F. Extracellular fluid (ECF) spaces: When an animal has a cavitary effusion, it has an increased amount of extracellular fluid. It is useful to remember the three "spaces" of ECF.
   1. First space: intravascular fluid (plasma)
   2. Second space: extravascular interstitial fluid (extravasation of plasma fluid to cause interstitial edema is a second-space loss of fluid)
   3. Third space: extravascular fluid in body cavities or spaces (fluid within the alimentary tract is considered outside the body, not a third-space fluid)

PATHOGENESES OF CAVITARY EFFUSIONS

Effusions accumulate in pleural and peritoneal cavities when one or more pathologic processes (Table 19.4) cause increased entry of fluid into the cavity and/or decreased removal of fluid from the cavity. Except for ruptured organs, the pathogenesis of each effusion involves processes that cause fluid to leave blood or lymphatic vessels and accumulate in a body cavity. Different pathologic processes create different types of effusions. These processes are illustrated in Figure 19.3. The composition of the effusion provides evidence for the type of pathologic process that led to the effusion (Table 19.5).

Table 19.4. Cavitary effusions: Pathologic processes, mechanisms, and conditions or disorders that produce effusions[a]

Pathologic process	Pathologic mechanisms	Effusion	Conditions or disorders
Transudation	Altered hydraulic and oncotic pressures; impaired lymphatic drainage	Transudate, protein-poor	Cirrhosis (dogs),* protein-losing nephropathies,* protein-losing enteropathies, noncirrhotic portal hypertension (presinusoidal, sinusoidal)
		Lymphatic transudate	Damaged afferent lymphatic vessels, lymphatic obstruction (e.g., lymphangiectasia)
		Transudate, protein-rich	Congestive heart failure,* portal hypertension (postsinusoidal)
Exudation	Increased vascular permeability to plasma proteins	Exudate, infectious	Bacterial (many types, sources)* fungal (e.g., *Blastomyces, Histoplasma, Candida*), viral (FIP coronavirus), protozoal (e.g., *Leishmania, Toxoplasma*), or parasitic (*Mesocestoides*) infections
		Exudate, noninfectious	Neoplasms, foreign bodies, bile peritonitis, pancreatitis, steatitis, uroperitoneum, or ischemic necrosis due to a torsion or vascular lesions*
Hemorrhage	Leakage of blood from vessels	Hemorrhagic effusion, acute	Trauma to blood vessels, hemostatic defects, neoplasms, potentially anaphylaxis in dogs*
		Hemorrhagic effusion, chronic	Neoplasms, hemostatic defects (vascular damage or factor deficiencies), or trauma*
Lymphorrhage (lymphorrhea)	Leakage of lymph from lymphatic vessels	Chylous effusion or nonchylous lymphocytic effusion	Cardiac disease, trauma, diaphragmatic lesions or hernia, neoplasms, mediastinal lesions, lymphangiectasia, lymph node disease
Rupture of hollow organ or tissue	Leakage of urine from urinary bladder, urethra, or ureter	Uroperitoneum, secondary exudate	Trauma,* urolithiasis, or urinary tract neoplasms
	Leakage of bile from biliary tract	Bilious effusion, secondary exudate, noninfectious	Cholelithiasis, cholangitis, bile duct carcinoma, or cholecystitis
	Leakage of gastric, abomasal, or intestinal contents	Secondary exudate, infectious or noninfectious	Gastric: perforated ulcer, gastric carcinoma, or trauma Intestinal: perforated intestine, intestinal neoplasm, intestinal obstruction, or intestinal ischemia

* Relatively common disorder or condition

[a] During the development of an effusion, more than one pathologic process may contribute: a persistent chylous effusion elicits an inflammatory process so that exudation contributes to the effusion; bile itself contributes little fluid to an effusion, but the inflammation it induces elicits exudation of fluid, protein, and cells.

Table 19.5. Common features of effusions (not criteria for classifying effusions)[a]

	Physical features[b]	TP_{ref} (g/dL)	TNCC ($\times 10^3/\mu L$)	Microscopic features
Transudate, protein-poor (includes lymphatic)	Clear Colorless	< 2.0	< 1.5	Macrophages, neutrophils, few lymphocytes ± reactive mesothelial cells
Uroperitoneum, initially	Clear Colorless to yellow	< 2.0	< 1.5	Nucleated cells vary; urine crystals, sperm, or bacteria may be found
Uroperitoneum, hours–days[c]	Clear to cloudy Yellow, tan	Varies	Varies	Mostly neutrophils and macrophages; ± urine crystals and bacteria
Transudate, protein-rich[c]	Clear to cloudy Colorless to pale yellow	≥ 2.0	< 1.5	Neutrophils, macrophages, few lymphocytes, reactive mesothelial cells
Proteinaceous exudate, FIP or other vasculitides[c]	Clear to hazy Yellow	≥ 2.0	< 5.0	Mostly neutrophils and macrophages; pink protein stippling and crescents if very high [TP]; ± fibrin strands
Exudate, infectious (bacterial, fungal, protozoal, or other organisms)	Hazy to cloudy Yellow, tan	≥ 2.0	> 5.0	> 80 % neutrophils; occasionally many macrophages; bacteria may be in neutrophils and free (rarely in macrophages); fungi and protozoa may be free, in neutrophils, or in macrophages
Exudate, noninfectious	Hazy to cloudy Yellow, tan, green	≥ 2.0	> 2.0	> 70 % neutrophils or > 80 % neutrophils and macrophages; bile pigment or foreign material may be in macrophages, neutrophils, or free
Hemorrhagic effusion, acute[c]	Opaque Red	≥ 2.0	> 2.0	Abundant RBCs, blood WBC populations, few or no platelets
Hemorrhagic effusion, chronic[c]	Hazy to opaque Red	≥ 2.0	> 2.0	Abundant RBCs; macrophages containing RBCs, hemosiderin, or hematoidin
Neoplastic effusion, lymphomatous	Hazy to cloudy[d] Yellow, tan	≥ 2.0	> 5.0[d]	Neoplastic-appearing lymphocytes (few to many) with various percentages of other WBCs
Neoplastic effusion, not lymphomatous	Hazy to cloudy Yellow, tan	≥ 2.0	> 2.0	Nonlymphoid neoplastic cells: ↑ mast cells ± atypia, atypical histiocytes, atypical epithelial or mesothelial cells, other; other findings vary
Lymphocyte-rich effusion, not chylous or neoplastic	Clear to cloudy Colorless to yellow, tan	≥ 2.0	< 10.0	Many small lymphocytes ± reactive lymphoid cells, mixed with macrophages and neutrophils
Chylous effusion[c]	Clear to opaque Colorless to white	?[e]	< 10.0	Many small lymphocytes, macrophages and neutrophils; lipid in macrophages, neutrophils, or fluid residue

[a] The table contains common features of effusions. However, the pathologic process that creates an effusion, not the results of fluid analysis, determines the type of effusion.
[b] All effusions may have a pink to red tint because of the presence of RBCs or hemoglobin. The RBCs or hemoglobin may be present because of the animal's pathologic condition or could be due to blood contamination during sample collection.
[c] Each of these effusions have been classified as *modified transudates*, despite not forming by transudation.
[d] Lymphomatous effusions can also be of low cellularity; they are recognized by a population of atypical lymphocytes.
[e] The TP_{ref} value is unreliable (may be falsely increased) when the effusion contains lipid.

However, the evidence frequently is not diagnostic of a particular disorder, and thus other information (history, physical examination, effusion volume and distribution, imaging findings, or other laboratory data) is needed to interpret the analysis results and establish the specific pathogenesis of an effusion. Quantitative results of fluid analysis do not define an effusion or alone reveal its pathogenesis, so classification by TNCC and [TP_{ref}], as has been commonly done in veterinary medicine, is not recommended by the authors. However, this quantitative information can help determine the pathologic process or processes that created an effusion. The following sections describe the types and pathogeneses of effusions, but they do not define a classification system based on fluid analysis.

I. Transudates
   A. Pathogenesis
      1. Transudates typically accumulate in pleural and/or peritoneal cavities when there is an excess diffusion of solute-rich fluid from the vascular space because of alterations in hydraulic and/or oncotic pressures. Transudates may also accumulate when lymphatic drainage from a cavity is impaired (Figure 19.3). When alterations are systemic, multiple cavitary effusions may form.
      2. For effusions originating in most tissues, a transudate is protein poor ([TP_{ref}] < 2.0 g/dL), but transudation from a liver can create a protein-rich peritoneal transudate if normoproteinemia is present. If marked hypoproteinemia is present, then the transudate associated with hepatic transudation will have a lower [TP_{ref}] than if formed when the plasma [TP] was not decreased.
      3. The major factor that determines if there is a protein-poor or protein-rich transudate is the location of the transudation. If the increased hydraulic pressure is within capillary beds that are poorly permeable to proteins, then a protein-poor transudate forms. If the increased pressure is within sinuses or capillaries that are permeable to proteins, then a protein-rich transudate forms.
      4. A transudative pleural effusion is caused primarily by increased hydraulic pressure in the alveolar capillaries. Most of the pulmonary transudate remains in the lung (i.e., pulmonary edema), but it may flow into the pleural cavity because of damaged visceral pleura. Concurrently, venous congestion and the resultant increased hydraulic pressure in the caudal vena cava may reduce lymphatic drainage from the pleural cavity.
      5. Portal hypertension is a frequent contributor to the formation of peritoneal transudates and occasionally pleural transudates. *Portal hypertension* is a pathologic state in which the hydraulic pressure in portal blood vessels is increased by either increased portal blood flow or, more likely, increased resistance to portal blood flow.
         a. Blood enters the abdominal portal venous system from capillary beds in viscera (intestines, spleen, pancreas, stomach, and distal esophagus), travels to the liver via the portal vein and its branches in the portal triad, flows through the hepatic sinusoids (capillaries) of the hepatic lobule to the central vein, exits the liver via the hepatic vein, and enters the caudal vena cava. Lesions within or adjacent to these vessels or the heart may impair portal blood flow and increase hydraulic pressure within the portal system.
         b. Portal hypertensive disorders can be classified by overlapping systems.[27,28] The *anatomic system* is primarily based on gross anatomy (i.e., prehepatic, intrahepatic, or posthepatic) and relates to the flow of blood to, through, and from the liver. The *vascular system* identifies the location of the lesion relative to the hepatic sinusoids, i.e., presinusoidal (portal vein and its branches in the portal triad),

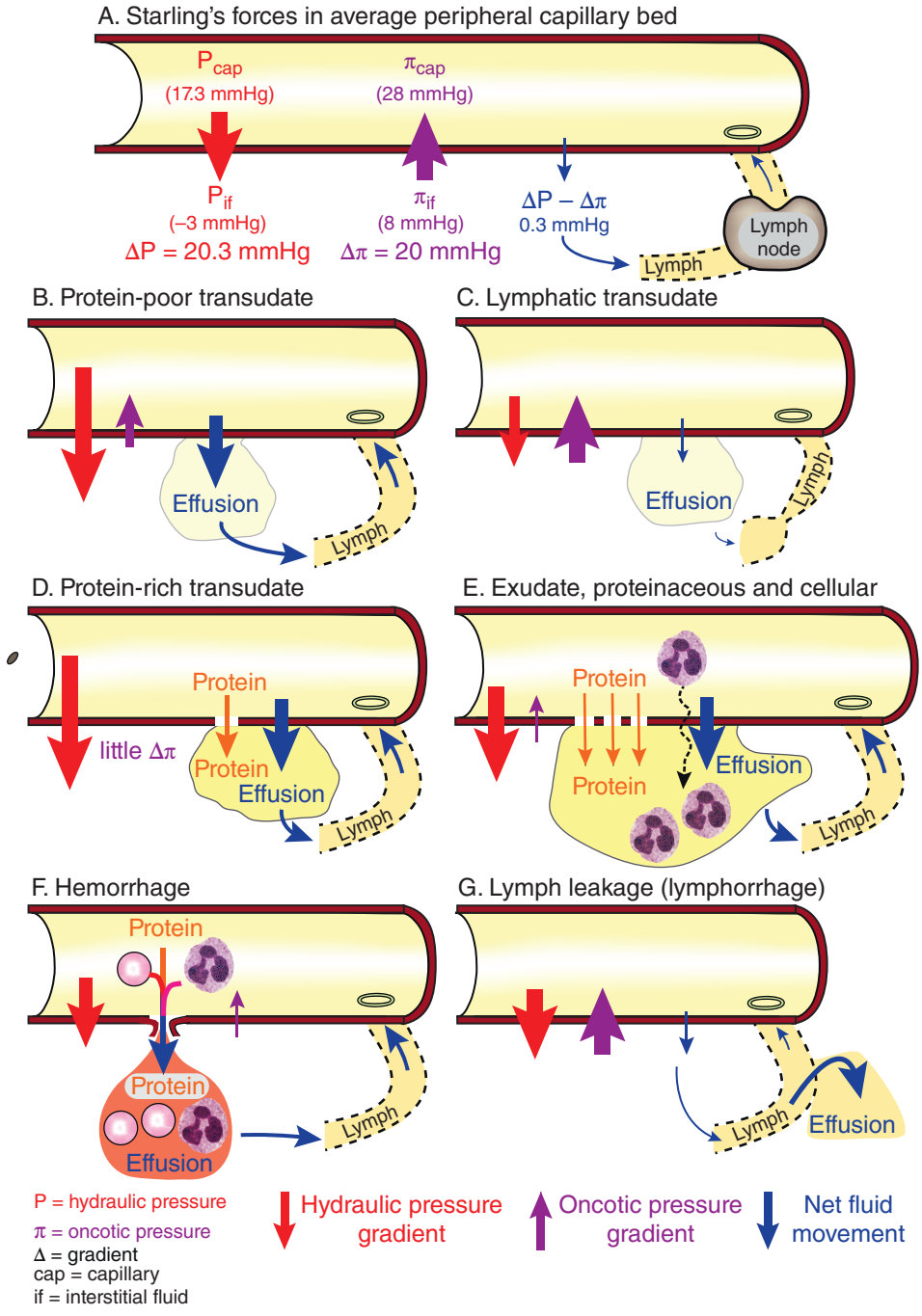


Figure 19.3. Major changes in Starling's forces of pleural and peritoneal effusions compared to Starling's forces during health. In each of the major effusions, the accumulation of fluid occurs because of an increase in the difference between ΔP and Δπ, i.e., ↑ (ΔP – Δπ).

A. Starling's forces in an average peripheral capillary bed in health (repeat of Figure 7.3 B): As explained in Figure 7.3, there are two opposite forces: (i) the hydraulic-pressure gradient (ΔP) promotes extravasation of protein-poor fluid, and (ii) the oncotic-pressure gradient (Δπ) promotes retention of the fluid in capillaries.

sinusoidal, or postsinusoidal (central vein to heart). The disorders that restrict portal blood flow can be classified as luminal (e.g., thrombosis) and extraluminal (e.g., hepatic fibrosis).

(1) Portal hypertension caused by right heart failure is posthepatic and postsinusoidal. Because there is increased hydraulic pressure in the hepatic sinusoids, the resulting effusion is protein rich.

(2) Portal hypertension caused by hepatic cirrhosis is intrahepatic but may be either presinusoidal or sinusoidal. When the increased hydraulic pressure is presinusoidal, the resulting effusion is protein poor. Sinusoidal hypertension may cause protein-poor transudation when hypoproteinemia is marked.

B. Protein-poor transudates and lymphatic transudates (Tables 19.4 and 19.5) (Figure 19.3B and C)

Figure 19.3. *continued*

Because the ΔP is slightly larger than $\Delta\pi$, the net result is fluid leaving the capillary blood. The fluid is removed by the lymphatic system and thus there is not an extravascular accumulation of fluid.

B. Protein-poor transudate: In the more common disorders (i.e., protein-losing nephropathy, hepatic cirrhosis, or other presinusoidal hypertensive states), there is an $\uparrow P_{cap}$ within portal capillaries because of hypertension created by the combination of Na^+ and H_2O retention (see Chapter 9). The $\uparrow P_{cap}$ creates $\uparrow \Delta P$ that tends to force more protein-poor fluid out of capillaries. If there is concurrent hypoproteinemia (especially hypoalbuminemia), as is common in these states, it reduces the π_{cap} more than the π_{if} to create a $\downarrow \Delta\pi$. The $\downarrow \Delta\pi$ reduces the inward draw of protein-poor fluid. The combination of more fluid leaving (due to $\uparrow \Delta P$) and less fluid returning (due to $\downarrow \Delta\pi$) overwhelms the drainage of the lymphatic system and thus a protein-poor transudate accumulates within cavities (third-space effusion) or in interstitial tissues (second-space effusion, edema).

C. Lymphatic transudate: When there is impaired drainage of interstitial fluid by lymphatic vessels, the fluid that normally enters the lymphatic system accumulates in a second space (edema) or in a third space (cavitary effusion). The lesions causing impaired drainage can be at lymphatic stomata, in lymphatic vessels, or in lymph nodes. If sufficient fluid accumulates, there is $\uparrow P_{if}$ and thus reduced ΔP.

D. Protein-rich transudate: When $\uparrow P_{cap}$ occurs in protein-permeable vessels, i.e., hepatic sinusoids or pulmonary capillaries, then $\uparrow P_{cap}$ creates an $\uparrow \Delta P$ that forces more protein-rich fluid out of blood vessels. Because these vessels are protein-permeable, there is little to no oncotic pressure gradient to pull the fluid back into the vessels. When the rate of fluid leaving vessels overwhelms the drainage of the lymphatic system, then a protein-rich transudate accumulates with peritoneal or pleural cavities (third spaces).

E. Exudate, proteinaceous and cellular: In an acute inflammatory response with increased blood flow to the inflamed tissue, $\uparrow P_{cap}$ creates an $\uparrow \Delta P$ that forces more fluid out of capillaries. Concurrently, inflammatory mediators increase the vascular permeability to proteins to allow proteins to enter the interstitial fluid (*represented by dashed vessel wall*). This escape of plasma proteins causes an $\uparrow \pi_{if}$ that reduces the $\Delta\pi$ and thus there is less fluid returned to vessels. The combination of $\uparrow \Delta P$ and $\downarrow \Delta\pi$ allows a protein-rich fluid to accumulate within the inflamed pleural or peritoneal cavities (third spaces). In most inflammatory states, chemoattractants within the body cavities induce the movement of inflammatory cells into the exudate to increase the TNCC. In vasculitides, inflammatory cells may not accumulate in the exudate because chemoattractants draw WBCs to vessels rather than to the body cavity.

F. Hemorrhage: A damaged blood vessel allows blood to escape into the pleural or peritoneal cavity to create a hemorrhagic effusion. Hemorrhagic fluid is protein-rich and thus its presence reduces the $\Delta\pi$ in the capillary beds. Thus, less of the fluid that normally leaves the capillaries returns to the vessels, thereby adding to the quantity of the effusion and diluting the protein and cell concentrations in the fluid. Concurrently, an active lymphatic system returns fluid, protein, and cells to blood (autotransfusion).

G. Lymph leakage (lymphorrhage): This type of lymphatic effusion occurs when pressure or other factors damage efferent lymphatic vessels, thus lymphocyte-rich fluid accumulates in extravascular spaces. If the fluid contains chylomicrons, then it forms a chylothorax or chyloabdomen.

1. Protein-poor transudates tend to form in body cavities when hypoproteinemia (including hypoalbuminemia) is marked. Most mammals with protein-poor transudates have a serum [albumin] < 1.5 g/dL. However, in most disorders, hypoalbuminemia by itself does not cause transudation.
   a. Analbuminemia occurs in people and is characterized by serum [albumin] < 0.1 g/dL; these people typically do not have transudative cavitary effusions but may have mild edema. Compensatory processes that prevent transudative effusions include increased synthesis of globulins, reduced intravascular hydraulic pressure, reduced interstitial oncotic pressure, and increased lymphatic drainage. The plasma oncotic pressure in analbuminemic people is about half of that found in healthy people.[29]
   b. The lack of significant transudative effusion in the absence of albumin emphasizes three concepts: (i) globulins contribute to oncotic pressures, (ii) increased lymphatic drainage of a cavity may compensate for increased transudation, and (iii) factors other than hypoalbuminemia (e.g., increased hydraulic pressure) typically contribute to transudative effusions.
   c. An acute onset of marked hypoproteinemia (e.g., marked blood loss followed by intravenous fluid therapy) may lead to transudation because there has been insufficient time for adjustments in the oncotic pressure gradient and lymphatic drainage.
2. The most common disorders that produce protein-poor transudates in some affected dogs are hepatic cirrhosis and protein-losing nephropathy (Table 19.4). Pathologic protein-poor transudates are uncommon in cats, horses, and cattle. With cirrhosis and protein-losing nephropathies, transudates form because of these two major factors:[26]
   a. Decreased plasma oncotic pressure
      (1) As the plasma [TP] falls, proteins (including albumin) in the interstitial fluid are redistributed to the vascular space. If the plasma [TP] and the interstitial fluid [TP] decrease by the same amount, then the oncotic pressure gradient does not change. Thus, additional fluid does not move from vessels. However, with marked hypoproteinemia, less interstitial fluid protein is available for redistribution to the vascular space, thus transudation is promoted.
      (2) When the oncotic pressure in plasma decreases more than that in the interstitial fluid, the oncotic pressure gradient is reduced, thus more fluid enters the interstitial space. However, a transudate will not accumulate if increased lymphatic drainage removes the fluid. In people, lymph flow can increase by at least a factor of 10 to compensate for an increased rate of fluid leaving plasma.[26]
   b. Increased hydraulic pressure gradient
      (1) In cirrhosis and protein-losing nephropathies, abnormal regulation of blood volume leads to retention of Na^+ and H_2O. The retention of H_2O causes an increased plasma hydraulic pressure and thus a greater rate of fluid leaving plasma and entering interstitium.[30] Portal hypertension also contributes to transudation in cirrhosis.
      (2) The processes that lead to the retention of Na^+ and H_2O involve hormones and renal functions (see Chapters 8 and 9).
3. Occasionally, lymph node diseases, protein-losing enteropathies, and idiopathic noncirrhotic portal hypertension cause the formation of protein-poor effusions.
   a. If a protein-poor effusion is associated with intrathoracic or intra-abdominal lymph node disease, at least part of the reason for the effusion may be decreased lymphatic drainage of fluids that normally enter the cavities (Figure 19.3C).
   b. If a protein-poor effusion is associated with a protein-losing enteropathy, at least two mechanisms may be contributing to the effusion: (i) if the enteropathy has led to

marked hypoproteinemia, then part of the pathogenesis is a decreased oncotic pressure gradient, and (ii) some protein-losing enteropathies impair drainage of intestinal lymph (e.g., lymphangiectasia).

c. Protein-poor transudates were reported in several dogs with either idiopathic hepatic fibrosis or chronic hepatitis, and in one dog with portal vein hypoplasia.[31] Presinusoidal portal hypertension was considered the cause of the transudation because the serum albumin concentrations were > 1.6 g/dL and therefore considered too high to explain the effusions by decreased plasma oncotic pressure. However, most dogs in the report were hypoproteinemic and hypoalbuminemic, and thus decreased oncotic pressure gradients probably contributed to transudation.

d. Idiopathic noncirrhotic portal hypertension in dogs is an uncommonly recognized disorder that may be caused by portal vein hypoplasia. Dogs with this disorder have protein-poor peritoneal transudates and many other laboratory data suggestive of a portosystemic shunt or hepatic insufficiency.[32]

4. It has been suggested that feline perinephric (perirenal) pseudocysts may rarely rupture and form transudate-like pleural effusions by drainage of cystic fluid from the retroperitoneal space to the pleural space via the mediastinum.[33] The ratios of fluid [Crt] to serum [Crt] in the two reported cats were 1.04 and 1.00, values not supportive of urothorax, and analysis of fluid from intact feline perinephric pseudocysts has shown that an affected cat's fluid [Crt] approximates its serum [Crt].[34,35] The mechanism by which this subcapsular transudate-like fluid accumulates is unclear. After partial capsular resection of these pseudocysts, peritoneal effusions of similar fluid can develop.[35]

C. Protein-rich transudates (Tables 19.4 and 19.5) (Figure 19.3D)

1. Protein-rich transudates typically occur when there is increased plasma hydraulic pressure in the liver or lungs because of venous congestion. Although the term *pure transudate* is often used to describe protein-poor transudates, these effusions can form by only passive transudative means and therefore may also be considered pure transudates despite their protein content.

2. The most common disorders that produce protein-rich transudates are congestive heart failure and portal venous hypertension (Table 19.4). Protein-rich transudates similarly form secondary to Budd–Chiari syndrome, which is veno-occlusive disease caused by congenital vascular defects or acquired neoplastic or nonneoplastic (e.g., thrombi, traumatic constrictions) obstructions to venous flow between the liver and the junction of the cranial vena cava with the right atrium. In all these conditions, transudation occurs because of increased plasma hydraulic pressure, and the [TP_{ref}] of peritoneal effusions is commonly 2.5–4.5 g/dL.

a. In congestive heart failure, a complex set of events decreases cardiac output, increases hydraulic pressure in veins, and increases Na^+ and H_2O retention (see Chapter 9).

b. In portal venous hypertension (posthepatic or postsinusoidal), the rate of plasma entering the space of Disse increases because of the increased hydraulic pressure in hepatic sinusoids. Very small increases in venous pressures cause protein-rich fluid to pass into the space of Disse, into hepatic interstitial fluid, and "sweat" from the hepatic surface to the peritoneal cavity.[36] The exact path of the protein-rich fluid from the space of Disse to the peritoneal cavity is not well defined. Some protein-rich fluid leaves the liver via lymphatic vessels. Other fluid might escape the hepatic lymphatic network and pass through Glisson's capsule; if so, the protein-rich fluid in the peritoneal cavity could be considered another form of a lymphatic transudate (see below).[37]

c. In people with these disorders, a serum albumin–ascites gradient (serum [albumin] – effusion [albumin]) is used to help differentiate effusions caused by portal

hypertension, e.g., cirrhosis and congestive heart failure, from other effusions. This gradient is greater in these congestive disorders than in exudative disorders.[38–40] Very little information about this gradient is available for domestic mammals (see Selected Analyses for Pleural and Peritoneal Effusions, Section X).

3. The [TP] of a transudate may increase when an animal is given a diuretic agent to reduce edema or the quantity of an effusion, because H_2O leaves the fluid faster than protein leaves it.

II. Exudates (Tables 19.4 and 19.5) (Figure 19.3E)

A. An exudate forms when inflammation causes increased vascular permeability that allows plasma (and its proteins) to ooze out of the blood. The exudation of protein-rich fluid is usually accompanied by the migration of WBCs (mostly neutrophils) into the effusion because of chemotactic substances in the fluid. However, high protein and nucleated cell concentrations do not alone indicate an effusion is an exudate or primarily formed by exudation. Hemorrhage, for example, is not exudation and a hemorrhagic effusion is not an exudate. Chylous and lymphomatous effusions do not form primarily by exudation, but they may have protein and cell concentrations similar to exudates in the absence of significant inflammation.

1. With exudation, vascular permeability is typically increased by the effects of inflammatory mediators (e.g., histamine, bradykinin, leukotrienes, and substance P) that were released from inflamed tissue. The inflammatory mediators may also cause selective vasodilation, so that more blood enters the inflamed tissues and thus increases the hydraulic pressure in the capillaries. In septic patients, a cascade of cytokine and inflammatory mediators may lead to systemic vascular damage and widespread increases in vascular permeability.
2. The inflammation can directly involve blood vessel (vasculitis and phlebitis), and then the vessels become very permeable to plasma proteins.

B. Neutrophils are the most common nucleated cell in most exudates, but mixtures of predominantly neutrophils and macrophages are common, and mixtures of predominantly neutrophils, macrophages, and lymphocytes occur. Occasionally, nucleated cells of exudates are predominantly eosinophils.

C. Exudates are caused by infectious and noninfectious agents (an etiologic agent of inflammation is not necessarily an organism). The type of exudate (i.e., infectious or noninfectious) cannot be reliably determined until the causative agent or event has been established, and that may require more than routine fluid analysis. Therefore, "nonseptic exudate" may be an inappropriate and misleading interpretation of an exudate when no organisms are seen microscopically.

1. Exudates are frequently caused by an infection with bacteria or other organisms. Some people consider *septic* and *bacterial* to be synonyms, but *sepsis* refers to any microbial infection (e.g., bacterial, fungal, viral, or protozoal). To reduce the possibility of confusion, it may be better to refer to the infectious exudates by the appropriate infectious agent (e.g., bacterial exudate or fungal exudate), when known. Although the presence of organisms indicates an infection, the underlying disorder may not be apparent.
   a. Organisms can reach a body cavity (i) hematogenously, (ii) from an external penetration, (iii) from an infected internal organ, or (iv) from the alimentary tract secondary to rupture, translocation through devitalized tissue, or penetration by a foreign body, including ingested wire or sewing needles.

b. If a peritoneal exudate contains mixed bacteria with many bacilli, an intestinal origin should be considered. In contrast, a single population of cocci in an equine pleural or peritoneal exudate would, respectively, suggest extension from streptococcal pleuropneumonia or an abdominal abscess. Note: A pleural effusion formed secondarily to a bacterial or other type of pneumonia is a *parapneumonic effusion* that can be classified more specifically based on the findings, e.g., neutrophilic and proteinaceous exudate with bacterial sepsis.

2. Exudates are also commonly formed during noninfectious inflammatory responses. The inflammatory response may be due to necrotic tissue (e.g., secondary to ischemia or neoplasia), the presence of a sterile foreign body (e.g., surgical sponge or barium), the presence of an irritating fluid (e.g., bile or urine), or even the presence of sterile saline or povidone-iodine in lavage fluid.[41] Peritoneal fluid analysis after equine abdominal surgery or open castration of stallions has shown marked increases in TNCC (mostly neutrophils) and increases in other inflammatory markers that may begin within 12 h and persist for over a week.[42,43]

D. When an effusion is protein rich and has a high [neutrophil], it is not difficult to recognize it as an exudate. However, it may be difficult to determine the cause of an exudate.

1. With bacterial and fungal exudates, microscopic examination of the fluid may detect bacteria (cocci or bacilli) or fungal structures (yeasts or hyphae) in phagocytes (neutrophils or macrophages) or free. However, if very few organisms are in the fluid, they may not be detected during the microscopic examination, and the fluid should be placed in appropriate culture media to attempt to grow organisms.
2. With the exception of the exudate of FIP, viral exudates are uncommon. The spectrum of proteins in FIP exudates is similar to that of plasma proteins because the FIP vasculitis enables plasma proteins to ooze through the inflamed vessel walls. Compared to most exudates, the FIP exudate tends to have a low nucleated cellularity because the inflammatory process is in the blood vessels and not in the body cavity. Microscopic examination of a stained preparation may reveal a pink granular residue or protein crescents reflective of a high [TP], but these findings are not specific for FIP.
3. Uncommon causes must be kept in mind. Protozoal exudates are uncommon in the United States, but amastigotes or tachyzoites can be found in phagocytes.

III. Hemorrhagic effusions (Tables 19.4 and 19.5) (Figure 19.3F)

A. When the primary reason for an effusion is hemorrhage, the fluid can be considered a *hemorrhagic effusion* causing hemoabdomen, hemothorax, or hemopericardium, depending on the location. Mild to marked hemorrhage can be caused by neoplastic or nonneoplastic vascular trauma or other defects in hemostasis. In addition, anaphylaxis has been the suspected cause of transient hemorrhagic peritoneal effusions in dogs, but the mechanism is not clear.[44] Relatively minor hemorrhage is a component of many exudates because of the vascular damage associated with inflammation. Also, minor hemorrhage commonly occurs during the collection of an effusion; this is commonly called a *traumatic tap* or *iatrogenic hemorrhage*. When recent hemorrhage causes an effusion, the collected fluid has many features of the animal's blood. Lymphatic vessels resorb the effusion's H_2O, solutes, and RBCs. Thus, the effusion volume and its composition change with time (see Routine Analysis of Pleural and Peritoneal Fluid, Section VI.C.6). About 65 % of RBCs are resorbed (autotransfused via lymphatics) within 2 d and 80 % within 1–2 wk.[45]

B. When an effusion forms because of frequent but small amounts of hemorrhage, the effusion can have a variety of features:

1. If collected soon after hemorrhage, it might have features of dilute blood.
2. With persistent hemorrhage, the extravasation of plasma proteins disrupts the oncotic pressure gradient, and thus H_2O tends to diffuse into the cavity and dilute its contents. Concurrently, H_2O and proteins are returning to plasma via lymphatic vessels.
3. Some RBCs are engulfed by macrophages to form erythrophages, and some macrophages degrade hemoglobin to form hematoidin (bilirubin) or hemosiderin. Hematoidin crystals may be extracellular or within macrophages; hemosiderin is found within macrophages but can be extracellular if macrophages have been damaged.

C. It is difficult to set a decision threshold for the classification of hemorrhagic effusion because many factors can alter the effusion's Hct (or hemoglobin or RBC concentrations), and sometimes an effusion develops because of hemorrhage plus other processes. If the effusion's Hct is > 3 %, then hemorrhage is contributing to the effusion; an effusion Hct of 5 % and a blood Hct of 25 % suggests that 20 % of the effusion's volume is blood.

D. Occasionally, an abdominal sample is collected from an enlarged spleen. The bloody sample may have a Hct > 3 %, but it does not represent a hemorrhagic effusion. Both splenic aspiration and splenic rupture should be considered when hematopoietic cells are present in an attempted abdominocentesis sample but not in the patient's blood.

IV. Lymphatic effusions (Tables 19.4 and 19.5)

A. Effusions caused primarily by lymphatic abnormalities can be classified as lymphatic effusions, but their pathogenesis and composition vary. The accumulating fluid may be the interstitial fluid that never entered lymphatics because of decreased drainage, or it may be primarily lymph that escaped from lymphatics. Composition further varies with the source of lymphatics and whether they are afferent or efferent relative to lymphoid tissue.

B. There is not a common term for the loss of lymph into a body cavity. Two terms that occasionally are used are lymphorrhage and lymphorrhea. *Lymphorrhage* is similar to *hemorrhage*, i.e., *lymphorrhage* is a process that results in lymph escaping from lymphatic vessels, just as *hemorrhage* is a process that results in blood escaping from blood vessels. *Lymphorrhage* is sometimes used synonymously with *lymphorrhea* but is also used to refer to a focal accumulation of lymphocytes in tissues. *Lymphorrhea* more commonly refers to the external loss of lymph from damaged lymphatic vessels. Occasionally, authors also use the term to refer to the processes that cause lymphedema or lymph-rich effusions.

C. Several pathologic states may cause lymphatic effusions. The pathogeneses of these states can be divided into two groups: traumatic and nontraumatic.[46]

1. In the traumatic group, physical damage to lymphatic vessels may allow lymph to enter the body cavity or impair the drainage of physiologically formed fluid, thus allowing it to accumulate.
2. In the nontraumatic group, lymphatic effusions occur when one or more of these mechanisms are present: (i) lymph stasis, (ii) lymphatic hypertension, (iii) defective lymphatic valve function because of dilated lymphatic vessels, and (iv) increased permeability of lymphatic vessels. Also, the persistence of a nontraumatic process might weaken a lymphatic vessel and cause a lymphatic vessel to leak or rupture. Clinical findings suggest that many chylous pleural effusions are caused by obstruction of the thoracic duct or cranial vena cava; contrast lymphangiography reveals extensive lymphangiectasia of mediastinal and pleural lymphatic vessels.[47]
3. Blockage of lymphatic vessels by neoplastic cells can be a major reason for effusions associated with malignancies. However, enhanced vessel proliferation and increased blood vessel permeability may also contribute to these effusions.[48]

D. Lymphatic effusions can be classified into these three major groups based on their cellular content and the presence or absence of chylomicrons in the effusion.
   1. Chylous effusions (Figure 19.3G)
      a. A *chylous effusion* is produced when chylomicron-rich and lymphocyte-rich lymph leaks from lymphatic vessels; when the fluid enters the pleural or peritoneal cavities, it may produce a chylothorax or chyloabdomen (chyloperitoneum), respectively. When a chylous effusion is present, it indicates that lymphatic vessels somewhere between the small intestine and the thoracic vena cava are affected. Chylomicrons are formed in intestinal mucosal cells, enter intestinal lymphatic vessels via lacteals, and enter blood via the thoracic duct in the thoracic inlet.
      b. Leakage of chylomicron-containing lymph may be caused by one or more of the following mechanisms: (i) transudation secondary to increased hydraulic pressure in the cranial or caudal vena cava, (ii) lymphatic exudation caused by local inflammation, or (iii) loss through a defect in the lymphatic vessel wall.
      c. Disorders recognized to cause chylothorax in cats, dogs, and horses include neoplasms, cardiac failure from cardiomyopathy or cardiac anomalies, trauma, lung lobe torsion, and infections, but frequently it is idiopathic.[49–54] Each of the listed causes can produce other types of effusions, and lung lobe torsions can be a cause or a consequence of chylothorax (or other effusions).
      d. Chyloabdomen occurs rarely.[55,56] In a retrospective study involving dogs and cats, malignant neoplasms were frequently the primary pathologic disorder when there were bicavitary chylous effusions, i.e., concurrent chyloabdomen and chylothorax; cardiac disease and intestinal lymphangiectasia were the other major disorders.[56] Inflammatory disease and trauma could also impair lymphatic drainage from the intestine and cause or contribute to a chyloabdomen.
      e. Chylomicrons may be abundant and thus create a creamy white fluid. However, they may be present in low concentrations that do not cause turbidity and that may or may not be visible microscopically; in such cases, chemical assessment of fluid [TG] and fluid [cholesterol] or serum and fluid [TG] may be used to confirm their presence (see Selected Analyses for Pleural and Peritoneal Effusions, Section I).
      f. In at least one dog, increased pleural fluid was caused by administration of fluid and propofol into the pleural space secondary to a misplaced central venous catheter.[57] Because propofol is formulated as a TG-rich lipid emulsion, the fluid mimicked a chylous effusion grossly and biochemically.
   2. Nonchylous lymphocytic effusions (Figure 19.3G)
      a. A *nonchylous lymphocyte-rich effusion* is produced when lymph without chylomicrons leaks from lymphatic vessels and enters the pleural or peritoneal cavity; efferent lymph drainage is somehow impaired. The lesion may involve lymphatic vessels that are not in the drainage path from intestine to thoracic duct and thus do not contain chyle or chylomicrons. However, the absence of chylomicrons might indicate a lack of dietary intake of lipids (thus, the intestinal lymph did not contain chylomicrons) or that the chylomicrons (or TG) did not persist in the effusion. Note: If the lymphocytes were neoplastic, the effusion would be classified as a neoplastic or lymphomatous effusion.
      b. The same general mechanisms that can cause or contribute to chylous effusions can cause or contribute to nonchylous lymphocyte-rich effusions: (i) transudation caused by altered lymphatic pressures, (ii) lymphatic exudation caused by local inflammation, or (iii) loss through a defect in the lymphatic vessel wall.

c. These effusions are relatively common in cats and often associated with cardiac disease or neoplasms that alter lymphatic pressures and drainage. They do not have the unique features created by chylomicrons, including the chylous pattern of [TG] and [cholesterol]. Small lymphocytes are a major nucleated cell type that may or may not predominate; their proportion can decrease with secondary inflammation, including that associated with centesis for drainage.

3. Lymphatic transudates (Figure 19.3C)
   a. The term *lymphatic transudate* is not widely used but it describes the fluid that accumulates when damaged lymphatics fail to clear it or when afferent lymphatics leak their contents. This fluid forms physiologically by the imbalance between hydraulic and oncotic pressures across capillary beds, and it may accumulate if not returned to circulation via lymphatics.
   b. These effusions are typically protein-poor transudates. Routine analysis cannot distinguish them from protein-poor transudates formed by increases in hydraulic and decreases in oncotic pressures.
   c. As mentioned in the section on protein-rich transudates due to post-sinusoidal portal hypertension, that type of peritoneal effusion may form from hepatic lymph and thus may be a lymphatic transudate.
4. Pseudochylous effusions
   a. *Pseudochylous effusions* are fluids that grossly have a similar appearance to chylous effusions (e.g., white to cream colored, and cloudy or turbid) but do not contain chylomicrons and do not have a high [TG]. They have been recognized in people (rarely) and very rarely in domestic mammals. Effusions described as "pseudochylous" in older veterinary literature had features of chylous effusions.
   b. Pseudochylous effusions typically have a high [cholesterol] that might result from the degradation of cell membranes or the trapping of cholesterol-rich lipoproteins (e.g., low-density lipoproteins) in the body cavity. They are very rare in people and may require years to develop.[40] A hazy to opaque, cholesterol-rich effusion was reported in a dog with sclerosing encapsulating peritonitis.[58]

V. Effusions caused by the rupture of a hollow organ or other tissue (Tables 19.4 and 19.5)
   A. Leakage of urine from the urinary bladder, ureter, urethra, or kidney
      1. *Uroperitoneum* (uroabdomen) occurs when urine enters the peritoneal cavity subsequent to trauma, urolithiasis, or neoplasia. Attempts to expel urine forcefully from a distended urinary bladder can rupture it.
      2. Initially, the peritoneal fluid has features of urine and may resemble a protein-poor transudate based on routine analysis (see Selected Analyses for Pleural and Peritoneal Effusions, Sections II and III), but it is not a transudate because it did not form by transudation. When the presence of urine in the peritoneal cavity initiates an inflammatory response, the effusion may have features of an exudate as well as a uroperitoneum. The TNCC varies from quite low (because of the relatively large amount of urine entering the cavity) to quite high when WBCs (mostly neutrophils) are chemotactically attracted by bacteria (in cases with urinary tract infections) or other inflammatory stimulants. The [TP_{ref}] usually remains < 2.5 g/dL.
      3. *Urothorax* caused by urine-containing fluid in the pleural cavity has developed in association with uroperitoneum, usually in the presence of a diaphragmatic defect.
   B. Leakage of bile from the biliary tract
      1. Several pathologic states may damage the biliary tract (ducts or gallbladder) sufficiently for bile to enter the peritoneal cavity and cause a *bilious effusion* (choleperitoneum). Disorders include cholelithiasis, necrotizing inflammation, neoplasms, trauma, and mucocele formation. Rarely, biliary fluid accumulates in the pleural cavity and causes a

bilious effusion, a condition that has been called *bilothorax* (biliothorax, cholethorax). Causes include trauma with diaphragmatic injury, biliary–pleural fistulas, and bile peritonitis, even when the diaphragm appears to be intact.[59] The [bilirubin] of bilious effusions is typically, but not always, greater than the animal's serum [bilirubin] (see Selected Analyses for Pleural and Peritoneal Effusions, Section VII).

2. The bile volume is not sufficient to create an excessive amount of peritoneal fluid, but the bile initiates inflammation (often low to moderate grade), which then creates an exudative effusion (bile peritonitis).

C. Leakage of esophageal, gastric, abomasal, or intestinal contents
1. Several pathologic states may damage the alimentary tract sufficiently for luminal fluid to enter the peritoneal cavity or, in cases of esophageal rupture or diaphragmatic hernia, the pleural cavity.
2. The luminal fluid volume may or may not be sufficient to create an excessive amount of peritoneal or pleural fluid. However, the fluid's content (e.g., bacteria and ingesta) initiate inflammation that then creates an exudative effusion.
3. Peritoneal effusions associated with acutely ruptured alimentary tracts may have low TNCC and [TP]. Phagocytized bacteria may be present and would be indicative of inflammation and bacterial sepsis despite low TNCC and [TP].

D. In mares, parturition-associated uterine ruptures and vaginal tears involving the peritoneal cavity may cause neutrophilic and proteinaceous peritoneal exudates with or without hemorrhage, commonly with cytologically detected bacterial sepsis.[60,61] Grossly, the fluid may therefore appear dark orange to red (from RBCs) and turbid (from increased TNCC and RBCs), with fewer inflammatory cells initially. In contrast, the TNCC of peritoneal fluid is minimally affected by uncomplicated natural and oxytocin-induced foaling, with TNCCs remaining less than 5,000/μL in one study.[61] Similarly, in that study, the peritoneal fluid of most clinically healthy post-dystocia mares had little change in [TP_{ref}] and TNCC, exceptions being moderately increased [TP_{ref}] to 3.1 and 3.4 g/dL and mildly increased TNCCs of 13,000/μL on day 1 and 16,300/μL on day 2 after parturition.

VI. Effusions caused by multiple processes

A. Many cavitary effusions accumulate because of multiple concurrent processes. For example, an abdominal neoplasm may have necrotic areas that result in an inflammatory reaction that causes an exudate. Concurrently, blood vessels may be damaged sufficiently to cause hemorrhage into the cavity. Also, neoplastic cells may enter lymphatic vessels and impair lymphatic drainage. Therefore, the animal may be presented with an effusion that forms because of exudation, hemorrhage, and impaired lymphatic drainage.

B. Other examples of multiple pathogeneses include the following:
1. Persistent protein-rich transudation in heart failure modified by a low-grade inflammatory reaction and exudation
2. Urine leakage in uroperitoneum and subsequent exudation caused by peritonitis
3. A lymphomatous (neoplastic) effusion with lymphatic damage causing a concurrent chylous effusion

ROUTINE ANALYSIS OF PLEURAL AND PERITONEAL FLUID

Analysis of pleural and peritoneal fluid may yield a fairly specific cause of the effusion (e.g., lymphoma, intestinal rupture, bile peritonitis, fungal infection), a more general cause of the effusion (e.g., inflammation, transudation, hemorrhage), or less specific information that is still useful because it provides information about the fluid's composition and potential pathogenesis. For example, with routine analysis, many effusions can be classified only as *protein-rich effusions*, which may be either

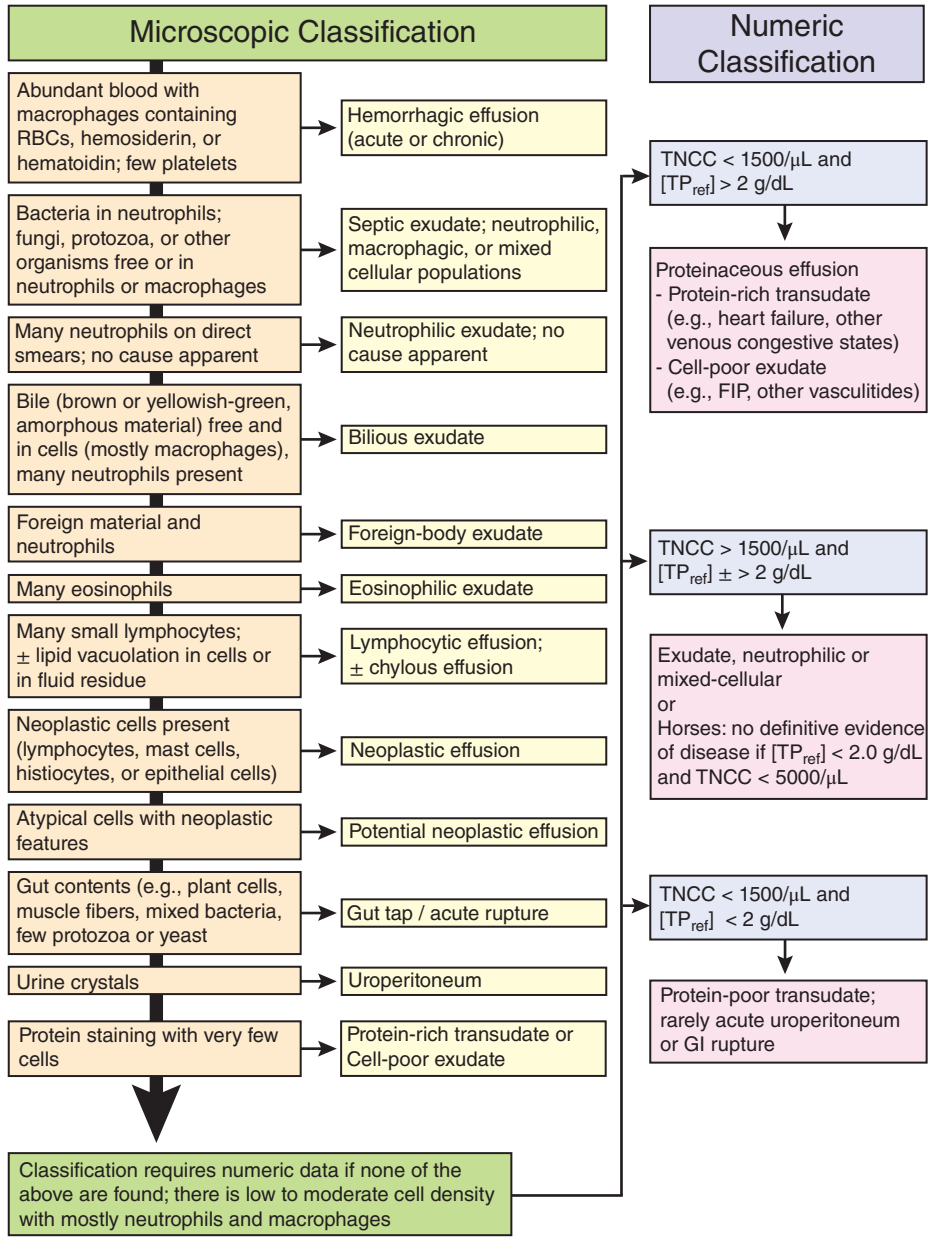


Figure 19.4. Effusion classifications based on microscopic and numerical criteria. Microscopic evaluation is necessary to completely classify cavitary effusions because it yields information that would otherwise be undetected, including specific diagnostic clues such as organisms and abnormal cell populations. Microscopic analysis may alone be sufficient for classification of a fluid as one of the following (*from top to bottom, left side*):

- Hemorrhagic effusion: All or some of the noted features may be present with the effusion Hct > 3 %; one must differentiate from iatrogenic hemorrhage during sample collection.
- Septic exudate: There is usually neutrophilic or neutrophilic and macrophagic inflammation; consider sample contamination if organisms are only extracellular.
- Neutrophilic exudate: Organisms may be present but not seen; consider submitting a sample for culturing, but also consider noninfectious causes.
- Bilious exudate: It may be difficult to find small amounts of bile pigment; consider submitting samples to compare [bilirubin] in paired effusion and serum samples.

proteinaceous exudates (inflammatory disease) or protein-rich transudates as are typical of cardiogenic ascites. Although this classification indicates neither a specific cause nor a pathogenic mechanism, it limits the options. Accurate classification would require further testing or clinical information that may or may not be available.

If appropriate historical and physical examination findings are known, results of many fluid analyses can be largely interpreted with the following three results: (i) [TP_{ref}], (ii) TNCC, and (iii) types and percentages of nucleated cells. However, TNCC and [TP_{ref}] alone can be misleading, and other features of an effusion may be necessary to determine its cause. These include microscopic findings (e.g., organisms, foreign material, bile), LD activity, results of other biochemical tests (e.g., [Crt], [TG]), other laboratory information (e.g., serum or plasma [TP]), and clinical information (e.g., the patient is in heart failure or is suspected of having a ruptured bladder). A flowchart can be helpful when a person is trying to establish the pathogenesis of an effusion. An example of a flowchart that focuses primarily on microscopic evaluation and secondarily on numerical TNCC and [TP_{ref}] is provided (Figure 19.4). This approach emphasizes that microscopy can provide more specific and differentiating information than is provided by TNCC, [TP_{ref}], serum [TP], and LD activity. For example:

- With [TP_{ref}] < 2 g/dL and TNCC < 2000/μL, an effusion could be a protein-poor transudate, a lymphatic transudate, a neoplastic effusion, or a ruptured viscus (bladder, stomach, intestine) with

Figure 19.4. *continued*

- Foreign-body exudate: Foreign material can be suture material, gauze, barium, injected material, or from a penetrating wound; if not detected or not present in the collected sample, the fluid would probably be classified as a neutrophilic exudate.
- Eosinophilic exudate: This indicates response to an allergen, parasite, or neoplastic tissue.
- Lymphocytic effusion: This typically reflects lymphorrhage from efferent lymphatic vessels; the fluid is often not transparent after centrifugation (chylous) but may be clear (chylous or nonchylous); TG and cholesterol assays may be considered for further characterization.
- Neoplastic effusion: More specific terms are used when cell types are identified (e.g., lymphomatous, carcinomatous); potential neoplastic cells must be differentiated from reactive mesothelial cells.
- Potential neoplastic effusion: A neoplasm is suspected but cells are not clearly neoplastic and may be reactive mesothelial cells; other diagnostic methods are needed to establish a diagnosis.
- Gut tap / acute rupture: One may not be able to differentiate a gut tap from an acute rupture; consider a repeat abdominocentesis.
- Uroperitoneum: Urine crystals are rarely seen in confirmed uroperitoneal fluid; carbonates and oxalate crystals may be seen in equine uroperitoneal fluid.
- Protein-rich transudates or cell-poor exudates: When a fluid's [TP] is great enough, protein crescents and stippling (precipitates) may be present and indicative of a protein-rich fluid; when these are prominent, the fluid is likely a proteinaceous exudate (e.g., FIP effusion).

If microscopic findings alone or in conjunction with other analytical findings do not establish an effusion classification, then the fluid has relatively low cellularity with little blood and mixed nucleated cells that are mostly neutrophils and macrophages. For such fluids with no distinguishing cytologic features, [TP_{ref}] and TNCC are needed for classification (*see Numerical Classification on the right, bottom to top*):

- When neither TNCC nor [TP] is clearly increased, the fluid is likely a protein-poor transudate, but clinical information should be considered to exclude acute gastric or urinary bladder ruptures that could yield similar findings. Rarely, neutropenia or hypoproteinemia could be present and limiting the increases in TNCC or [TP] expected with other effusions.
- When TNCC and [TP] are greater than expected in health, the fluid can be classified as an exudate (relatively low grade). In healthy horses or horses without clinical disease, the TNCC is commonly high enough to suggest at least low-grade inflammation in other species.
- When the TNCC is not clearly increased but the [TP] is, the fluid is best classified nonspecifically as a proteinaceous or protein-rich effusion; it may be either a protein-rich transudate or a proteinaceous exudate (inflammatory). This is a common type of fluid. Effusion LD activity or a serum albumin-effusion gradient may be helpful.

or without sepsis. Microscopy might detect neoplastic cells, a predominance of lymphocytes, or evidence of a ruptured organ.

- With [TP_{ref}] ≥ 2 g/dL and TNCC > 2000/μL, an effusion could be an exudate, a neoplastic effusion, a chylous effusion, or a hemorrhagic effusion. Microscopy might detect organisms, neoplastic cells, a predominance of lymphocytes and evidence of chylomicrons, evidence of pathologic hemorrhage, or evidence of a ruptured organ.
- With [TP_{ref}] ≥ 2 g/dL and TNCC < 2000/μL, an effusion could be a protein-rich transudate, a proteinaceous exudate, or a neoplastic effusion. Microscopy might detect neoplastic cells.
- In addition, [TP_{ref}] and TNCC can be atypically and misleadingly low when an effusion develops in a patient that is already hypoproteinemic (e.g., from intestinal protein loss) or neutropenic (e.g., from chemotherapy).

I. Sample collection and processing
   A. Sterile collection methods should be used to ensure that bacteria or other organisms are not introduced into the cavity. The centesis site and method vary among species: dogs and cats,[1,2,4,5] horses,[3,6,62] cattle,[18,20] and camelids.[21]
   B. Collected fluid should be placed into two tubes: one tube that contains EDTA, which inhibits fibrin clot formation, and one sterile tube (e.g., plain clot tube) for possible submission for microbiological testing (e.g., culturing) or chemical analysis. Tubes that are not immediately submitted to a laboratory should be kept cool and delivered to the laboratory within 36 h, although cell deterioration begins almost immediately. Concurrent submission of stained and unstained direct or concentrated preparations made from an aliquot of the fresh fluid can be very helpful. Some EDTA tubes may contain an additive that falsely increases the [TP_{ref}].[63] Heparin may be used as an anticoagulant, but it can alter sample staining and thus may interfere with microscopic analysis.
   C. Methods of preparing specimens for microscopic examination depend on the cellularity of the fluid. When possible, cytopreparations should be made soon after a sample is collected, so that in vitro changes are less likely (e.g., cell deterioration, organism proliferation, or in vitro phagocytosis). The fluid is air-dried prior to staining with routine stains. For all of the film methods, the feathered edge, which frequently contains important cells or structures, should be in the stainable area of the slide (the central two-thirds for one common type of automated stainer).
      1. *Direct smear*: Using the common wedge technique for making a blood film, a small drop of fluid is spread on a glass slide. This method may be adequate for effusions that have a TNCC > 5.0 × 10^3/μL, but an additional concentration method is desirable for fluids with a TNCC of up to at least 20.0 × 10^3/μL.
      2. *Line and stop-flow (flow-back) preparations*: These methods are similar to the direct smear, but the push slide is abruptly stopped and lifted so that cells are concentrated in a terminal line. This method tends to concentrate cells in a line, and can be used for fluids with a TNCC < 10.0 × 10^3/μL. However, a high [TP] tends to make this area too thick for a microscopic examination. This can be partially resolved by adding a flow-back step: when the spreader slide is lifted to form the terminal line, the slide is propped up until dry to allow the terminal line to flow back and spread out enough for better cell visibility. The cells along the leading edge of the flow-back smear are generally flattened the best, while cells in the flow-back area are more balled up but often recognizable, not lysed as is common at the ends of direct smears of low-cellularity fluid.
      3. *Sediment smears* are used for effusions with a low TNCC. This concentration method is not needed for effusions that have a TNCC > 20.0 × 10^3/μL.

a. Fluid is centrifuged in a clinical centrifuge by methods similar to preparing urine sediment. After removal of most supernatant, the sediment is resuspended in a small amount of fluid, and then a direct smear method is used to distribute cells on a glass slide.
b. Another method involves using simple devices to allow gravity to form a sediment slowly. This procedure may or may not create a monolayer of cells for microscopic examinations.

4. *Cytocentrifuge preparation*: Commercial cytocentrifuges are designed to concentrate cells in a fluid on a glass slide and are especially valuable for fluids that have a TNCC $< 1.0 \times 10^3/\mu L$. However, their cost limits their use to larger laboratories that analyze such fluids frequently. A Cytospin is a particular cytocentrifuge brand name, but the term is sometimes used generically (cytospin) as a synonym for cytocentrifuge.
5. Usually, the TNCC is not known prior to preparing cytopreparations, but it can be predicted based on the transparency of the fluid. If the fluid is clear or hazy, the TNCC is low, and thus a concentrating method is needed. If the fluid is cloudy or opaque, then a concentrating method is probably not needed. If the fluid is bloody, a concentration method may produce preparations in which nucleated cells are obscured by RBCs. When in doubt, both types of cytopreparations are made, and the microscopist can select the best slide for examination; preparation methods should be made clear.

II. Physical analysis
A. Color and transparency of the fluid (noted before centrifugation and, if the effusion is not clear and colorless, also after centrifugation)
1. A supernatant's color reflects the pigmented solutes in the fluid, such as bilirubin (yellow to orange), hemoglobin (pink to red to brown), stercobilinogen (brown), and chlorophyll (green). A nontransparent white, creamy, or cloudy supernatant typically indicates the presence of lipoproteins (e.g., chylomicrons) in the fluid. Combinations occur.
2. A sediment's color is an indication of the pigments in the suspended cells or particles in the fluid, e.g., red for RBCs, cream to beige or tan for nucleated cells, and brown for fecal content. The amount of sediment typically reflects the number of RBCs and nucleated cells in the effusion.
3. If clots are present, the $[TP_{ref}]$ is falsely decreased. If clots or other particulates are present, cell concentrations may be erroneous; the particulate material should be evaluated microscopically.

B. Refractometric estimates of [total protein] ($[TP_{ref}]$)
1. Clinical refractometers measure the refractive index of a fluid, which is then displayed on a TP, SG, or total solids scale; some current refractometers have "TS" in their names but have TP and not total solids scales. Most clinical refractometers are calibrated for the normal constituents of human plasma (TP scale) and human urine (SG scale). To reduce the effects of ambient temperature, temperature-compensated refractometers are recommended.
2. The [TP] scale is calibrated for plasma [TP] and approximates the true [TP] in protein-rich fluids because the altered refractive index is mostly due to changes in protein concentrations; concentrations of other dissolved solids of plasma are relatively constant. Even though the $[TP_{ref}]$ may not be accurate, the estimated [TP] is a differential feature of effusions.
3. Some clinical refractometers have a total solids (TS) scale with a unit of either "%" or g/100g, i.e., the grams of dissolved solid per 100 g of solution instead of grams per

Table 19.6. Conversion of refractive indices to [TP_{ref}], [TS_{ref}], total solids percentage (TS %), or specific gravity (SG) values[a]

Refractive index	Plasma or serum				Urine
	[TP_{ref}] (g/dL)	[TS_{ref}][b] (g/dL)	TS % (g/100g)	SG[c]	SG
1.3376	1.0	2.7	2.7	1.011	1.013
1.3384	1.5	3.1	3.1	1.012	1.015
1.3393	2.0	3.6	3.6	1.013	1.018
1.3402	2.5	4.2	4.1	1.014	1.020
1.3412	3.0	4.7	4.6	1.016	1.023
1.3421	3.5	5.2	5.1	1.017	1.025
1.3430	4.0	5.7	5.6	1.018	1.027
1.3438	4.5	6.1	6.0	1.020	1.029
1.3448	5.0	6.6	6.5	1.021	1.032
1.3457	5.5	7.1	7.0	1.022	1.034
1.3467	6.0	7.7	7.5	1.023	1.036

[a] This table includes data for [TP_{ref}] from 1.0 to 6.0 g/dL at 0.5 g/dL intervals. The table in the cited reference has [TP_{ref}] for each 0.1 g/dL from 0.0 to 14.1 g/dL. Because the relationships are nearly linear, other values can be estimated by interpolation.[64]

[b] [TS_{ref}] = TS % when the solutes are dissolved in H_2O, because 1 dL of H_2O weighs 100 g and proteins are not displacing H_2O. In plasma and serum, protein displaces H_2O and thus 100 mL of plasma may contain 93 mL or 93 % H_2O and 7 % protein (by volume). The nonprotein solids are more concentrated in the 93 mL H_2O because their concentrations are regulated in the H_2O component of the plasma.

[c] Values are rounded to the nearest thousandth so they can more easily be compared with the urine SG values.

deciliter (g/dL) of fluid. These yield different results because 100 mL of H_2O weighs 100 g, but 100 mL of plasma contains dissolved solids and weighs more than 100 g. Assuming plasma is about 93 % H_2O and contains no abnormal solute concentrations, plasma with a [TP_{ref}] of 6.0 g/dL has a [TS_{ref}] of 7.7 g/dL and a TS % of 7.5 g/100g (Table 19.6).

4. High concentrations of lipids (as seen in chylous effusions) increase a fluid's refractive index and produce falsely increased values for [TP_{ref}]; consequently, measurement of [TP_{ref}] in such fluids is not indicated. High concentrations of solutes other than proteins (e.g., urea in uroperitoneum) also falsely increase the TP_{ref} value.
5. Suspended cells do not interfere with refractometric values, because the cells are suspended particles and do not alter refractive index significantly (see the Physical Examination of Urine section in Chapter 8). Whenever in doubt, the refractometric value of fluid with suspended cells can be compared to that of the fluid's supernatant.
6. A variety of decision thresholds for [TP_{ref}] have been proposed for differentiating effusions. However, several factors determine an effusion's [TP_{ref}], so such decision thresholds should be considered as no more than suggested guidelines.
    a. A lower [TP_{ref}] (usually < 2.0 g/dL and frequently near 1.0 g/dL) is found primarily in protein-poor transudates, but also in effusions caused by urinary or alimentary tract rupture (prior to inflammatory response).

b. A [TP_{ref}] > 2.0 g/dL is found in most other effusions. Typically, the greater the [TP_{ref}] is, the greater is the protein permeability of blood vessels (or there is hemorrhage).
c. [TP_{ref}] in exudates from severely hypoproteinemic patients are lower than expected for exudates.

7. One study described a falsely increased [TP_{ref}] (e.g., about a 1.5 g/dL increase) when peritoneal fluid was added to an EDTA tube that also contained additives to prevent crystallization of the EDTA.[63] Not all commercial EDTA tubes contain the additive, and, by itself, the EDTA (at the recommended concentration) had minimal effect on the fluid's refractive index if the EDTA tube was at least one-third full. Refractometry can be used to assess EDTA tubes for their potential to falsely increase [TP_{ref}] values, i.e., by assessing a fluid refractometrically prior to and after adding different volumes of it to EDTA tubes.

C. Refractometric estimates of specific gravity (SG)
1. The SG scale is calibrated for urine and approximates the true SG of a protein-poor and glucose-poor fluid because most of the fluid's refractive index is due to electrolytes (therefore, similar to urine refractive properties).
2. Clinical refractometers do not have a scale for *plasma* SG, but conversion tables are available; examples of the conversion are provided in Table 19.6.[64] Note that the difference in the SG values (urine versus plasma) becomes greater as the [TP_{ref}] increases. Also note that the [TP_{ref}] is not equal to total solids (TS) concentration or total solids percentage, so one should be aware of the type of scale on a refractometer and refer to it accordingly (total protein versus total solids).
3. If a laboratory reports a SG for a body fluid, the value might be a "urine SG" (if taken from refractometer scale) or a "plasma SG" (if taken from a conversion table). Some published methods of classifying effusions use [TP_{ref}] and SG values. If a SG of 1.017 or 1.019 is a decision threshold, the SG value was from the urine SG scale. A refractive index of 1.3393 converts to a urine SG of 1.018 and a plasma SG of 1.013 (Table 19.6).
4. Using the refractometer to estimate urine SG is very useful because the SG values typically reflect changes in urine osmolality and thus provide an assessment of H_2O conservation by kidneys (see Chapter 8). However, plasma SG or osmolality values typically do not help with the interpretation of effusions.

III. Chemical analysis
A. As described in the previous section, an effusion's [TP] is routinely estimated by measuring its refractive index. A chemical assay to measure [TP] may be used, but the additional expense may not be warranted.
B. The concentrations of urea, Crt, cholesterol, TG, electrolytes, and other substances are occasionally measured to characterize some effusions (see the Selected Analyses for Pleural and Peritoneal Effusions section).

IV. [RBC] or hematocrit (Hct)
A. Either a [RBC] or a Hct should be determined when the effusion is pink to red, so that those values can be compared with blood [RBC] or Hct. The analytical methods used for measuring [RBC] or Hct in blood (see Chapter 3) typically can be used for effusions when the values are not lower than the assay's detection limit.
B. Effusions other than transudates typically contain a few RBCs either because the pathologic disorder is causing minor blood vessel damage or because the collected fluid was

contaminated with blood during collection. However, the [RBC] in most pink effusions is typically very low (Hct < 3 %, [RBC] < 0.5 × 10^6/μL); such values do not represent a hemorrhagic effusion.

C. When hemorrhage is a major contributor to the formation of the effusion, the [RBC] (or Hct value) can approach the values found in an animal's blood. Soon after hemorrhage into a cavity, RBCs and fluid are resorbed via lymphatics while altered oncotic pressure gradients promote fluid movement from interstitium to the body cavity and decrease the [RBC].

V. Total nucleated cell concentration (TNCC)

A. Because pleural and peritoneal fluids contain WBCs, mesothelial cells, and potentially other nucleated cells, it is appropriate to refer to their concentration as a TNCC instead of a [WBC]. However, the electronic and microscopic methods of determining WBC concentrations (see Chapter 2) provide adequate TNCC values for most fluids. If the nucleated cells are present in clumps or tissue fragments, the measured TNCC is less than the true concentration.

B. The hemocytometer method of measuring TNCC is usually recommended if the fluid is grossly abnormal, because the fluid may contain clumps of cells, debris, or other material that could plug the small tubing or orifices of electronic cell counters.

C. By itself, an effusion's TNCC has limited value for determining the cause of an effusion. The lowest TNCC values (frequently < 1.0 × 10^3/μL) are found with protein-poor transudates but may occur with sepsis. The greatest TNCC values (occasionally > 100.0 × 10^3/μL) are most often found with exudates but may occur with neoplastic lymphoid effusions.

VI. Microscopic examination

A. When cavitary effusions are clear and colorless (i.e., look like H_2O and therefore are protein-poor and cell-poor), a microscopic examination of the fluid often, but not always, yields little useful information. For all other effusions, the microscopic examination of stained cytopreparations is frequently the most important part of the fluid analysis. Part of the microscopic examination is the examination of cells, but examination of other structures and features is frequently important.

1. Stains designed for blood films are usually used for the routine staining of cytopreparations. One major advantage of using the blood stains is that people are familiar with the staining of blood cells and similar features are found in the nucleated cells of effusions.
2. Microscopic examination should include a low-magnification scan of all parts of one or more cytopreparations because of the potential uneven distribution of cells and other microscopic structures.

B. Major aspects of the examination are as follows:

1. A nucleated cell differential count is done to determine percentages of each nucleated cell type; the count is completed by either a subjective estimate or an objective enumeration. A hematology analyzer may provide useful differentials for fluids that contain mostly typical blood cells (not many large macrophages, mesothelial cells, or neoplastic cells). Reproducible microscopic enumeration often may require differentiation of more than 100 cells, particularly when there is substantial clustering of certain cell types. Interpretations should not be based heavily on the percentage of a certain cell type (e.g., reaching different conclusion with neutrophil concentrations of 70 % versus 85 %) without considering the imprecision in the value, which may be

substantial. With a measured TNCC and a nucleated cell differential count, approximate concentrations of each cell type can be calculated and interpreted; 60 % neutrophils in a fluid with a TNCC of $1.0 \times 10^3/\mu L$ is interpreted differently from 60 % neutrophils in a fluid with a TNCC of $100.0 \times 10^3/\mu L$. If cell concentrations are calculated from the percentages, they should be considered estimates because of inaccuracies of TNCC values and nucleated cell differential percentages.

2. Some people include mesothelial cells in the differential cell count, and others do not. If many individual mesothelial cells were present and included in a TNCC, then not including them in a differential count might result in erroneous estimates of cell concentrations and potentially an altered interpretation. However, mesothelial cells are often (i) a numerically insignificant population, (ii) large cells or in clusters and therefore at least partially gated out of inclusion for automated TNCCs and not all counted (when in clusters) for hemocytometer-derived TNCCs, and (iii) distributed unevenly in microscopic preparations leading to increased variability and inaccuracies of the differential and hemocytometer cell counts, depending on whether clusters of cells are present in the counting regions.
3. During the examination, diagnostic features of cells are identified: size, shape, nuclear features, and cytoplasmic features including inclusions or microorganisms.
4. If present, extracellular structures such as microorganisms and other material are identified.

C. Cells that are routinely identified in pleural and peritoneal fluids
   1. Neutrophils
      a. Generally, an increased [neutrophil] in an effusion indicates that exudation is a component of the effusion formation. However, exudation may be the primary reason for the effusion, or exudation may have occurred after another process created the effusion.
      b. The neutrophils in effusions may have microscopic features that are similar to those seen in blood films because they have not degenerated; if so, they are called *nondegenerate neutrophils* (Figure 19.5).

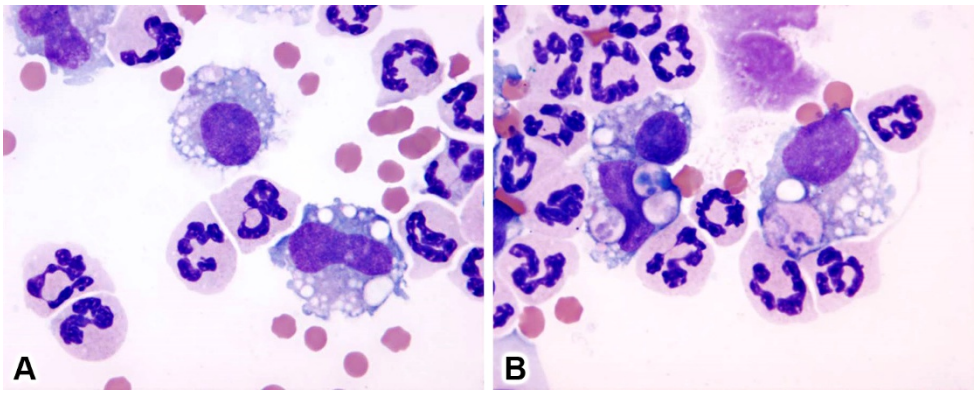


Figure 19.5. Nondegenerate neutrophils and macrophages (A and B); peritoneal fluid, horse. In B, two macrophages contain neutrophils at various stages of degradation within phagolysosomes. Mild collection-induced RBC contamination is common. Cytocentrifuge preparation, Wright stain, 100× objective.

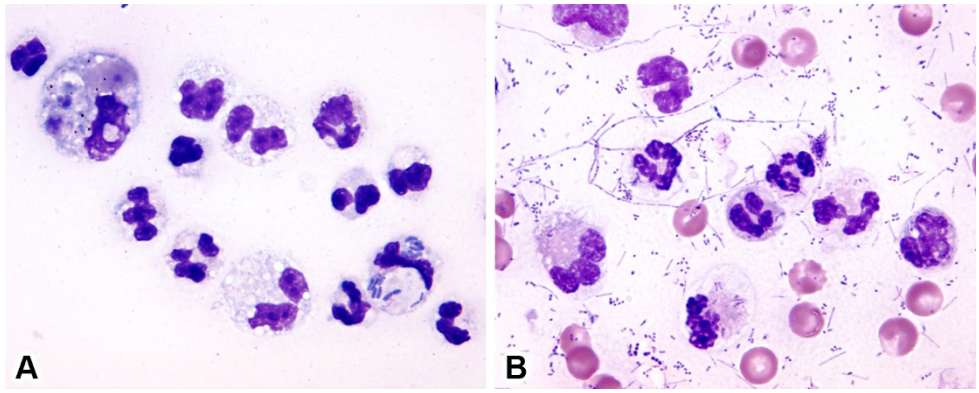


Figure 19.6. Degenerate neutrophils in exudates with bacterial sepsis, peritoneal (A) and pleural (B) fluid, dogs. A. All cells but the macrophage (*near upper left*) are neutrophils, one of which contains bacilli; degenerative changes include swollen nuclei and vacuolated cytoplasm. B. Mildly degenerate neutrophils with numerous bacteria, including branching, beaded, filamentous bacilli suggestive of *Actinomyces* sp. or *Nocardia* sp. Direct smears, Wright stain, 100× objective.

(1) The presence of nondegenerate neutrophils in body fluids occurs with nonbacterial and some bacterial exudates. Many neutrophils are cleared by macrophages in which they rapidly undergo lysosomal degradation after leukophagocytosis (Figure 19.5B).

(2) Segmented and nonsegmented neutrophils are generally not differentiated outside of blood. Degenerative changes and age-related deterioration alter nuclear features and can lead to inaccurate classifications. Whether immature or mature, neutrophils in effusions migrated from blood and indicate inflammation when they are increased.

c. Degenerate neutrophils are cells that have acquired certain structural defects after they have left the blood. Microscopic features of the classic degenerate neutrophil include a swollen and pale-staining nucleus, lack of nuclear chromatin patterns, and variable degrees of cytoplasmic vacuolation in intact cells (Figure 19.6).

(1) Because of many variations in neutrophil appearance from severely degenerate to nondegenerate cells, consistent classification of neutrophils as degenerate or nondegenerate is difficult. Iatrogenic cell damage (lysis) also impacts classification, though only intact cells should be classified.

(2) A degenerate neutrophil is not a toxic neutrophil. Toxic neutrophils develop their structural features in bone marrow and typically are reported only in blood or marrow samples even though they may be seen at inflammatory sites. Microscopic features of degenerate neutrophils reflect events that occur after neutrophils leave the vascular system.

(3) Nondegenerate neutrophils can deteriorate within hours of sample collection, and thus they may acquire features resembling degenerate neutrophils. Therefore, it may be difficult to differentiate in vivo degenerative change from in vitro deterioration if cytopreparations were not made soon after sample collection.

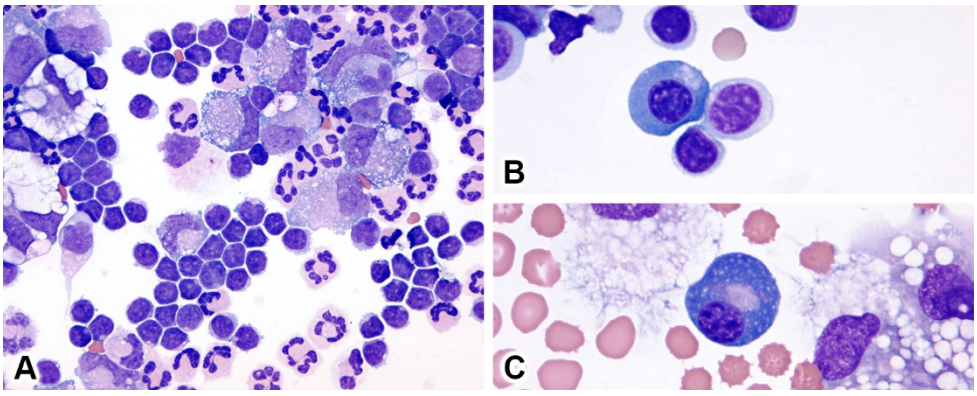


Figure 19.7. Lymphocytes and plasma cells, pleural fluid, cats (A, B) and dog (C). A. Chylous effusion with a predominance of small lymphocytes; fewer nondegenerate neutrophils and macrophages. B. A cluster of a small lymphocyte, an intermediate-sized lymphocyte, and a plasma cell; nuclei of small lymphocytes and plasma cells are of similar size and mildly wider than a RBC. C. A plasma cell with typical eccentric condensed nucleus, RNA-rich basophilic cytoplasm, and paranuclear clear area, adjacent to vacuolated macrophages. Cytocentrifuge preparations, Wright stain, 50× (A) and 100× objectives (B, C).

d. Differentiating nondegenerate from degenerate or deteriorated neutrophils is less important than searching for direct evidence of the cause of the inflammation because degenerate-appearing neutrophils may occur without sepsis and nondegenerate neutrophils may occur with sepsis. However, increased vigilance for organisms is appropriate when degenerate neutrophils are present.

2. Lymphocytes
   a. The lymphocytes in most effusions resemble the small lymphocytes that are typically seen in blood films. These cells have small nuclei (with diameters < 10 μm in areas where cells are well spread), clumped chromatin patterns, and small amounts of blue cytoplasm (Figure 19.7).
   b. When they have been stimulated, the lymphocytes may have features of reactive lymphocytes, plasmacytoid lymphocytes, or plasma cells (Figure 19.7B and C).
   c. Neoplastic lymphocytes (Figure 19.8) often have nuclei with diameters ≥ 10 μm and finely stippled to homogeneous chromatin patterns, but there are many variations. Frequently, the cells have moderate amounts of deeply basophilic cytoplasm, and the cells may have prominent or large nucleoli. Granular lymphocyte neoplasms may be characterized by cells with variously sized reddish cytoplasmic granules that are usually clustered in one region of the cytoplasm, mild to moderate cytoplasmic basophilia, and coarse to fine chromatin patterns.
   d. In some effusions, differentiating neoplastic from reactive (proplastic) lymphocytes can be difficult or impossible based on their microscopic features. Then, other diagnostic methods (e.g., assessment of an associated abnormal solid tissue mass, immunophenotyping) may be needed to determine whether the lymphocytes are neoplastic.
3. Macrophages
   a. Outside of the vascular system, monocytes transform into macrophages or histiocytes. Cells with the appearance of monocytes, macrophages, or transition

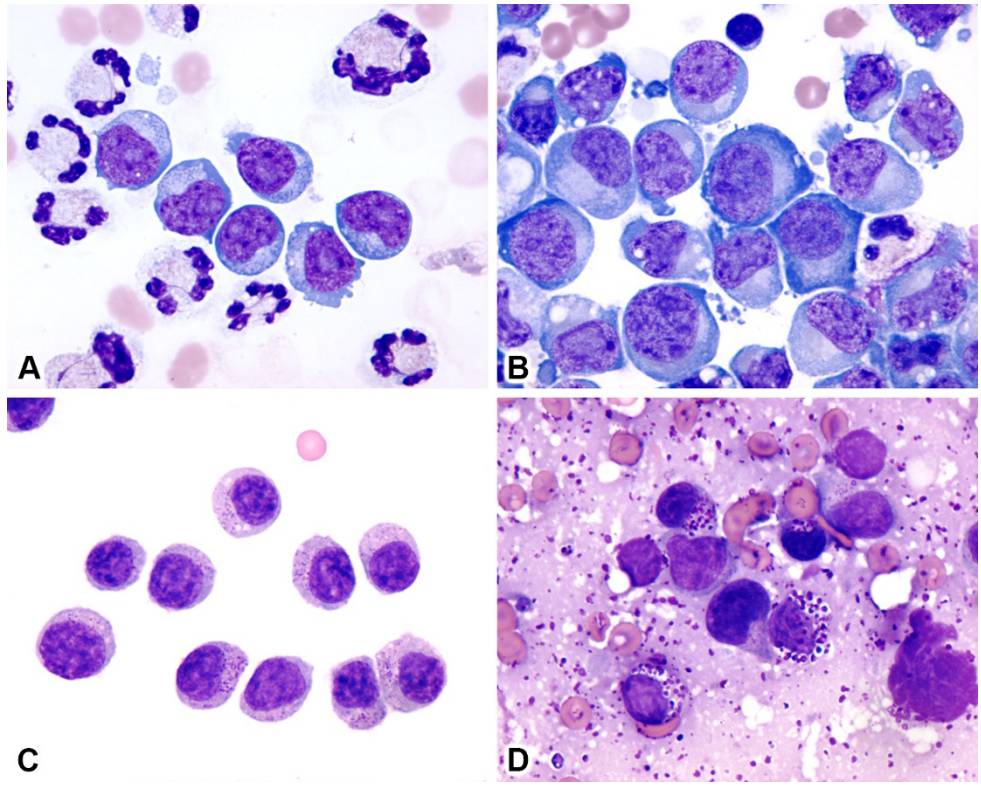


Figure 19.8. Lymphomatous effusions. A. Atypical intermediate-sized lymphocytes with increased cytoplasmic basophilia and large nucleoli are mixed with nondegenerate neutrophils, peritoneal fluid, dog. B. A predominating population of intermediate and large lymphocytes contrasts with a small lymphocyte (*top, center*) that has condensed chromatin and scant cytoplasm; pleural fluid, dog. C. Population of lymphocytes with fine reddish cytoplasmic granules (granular lymphocytes), peritoneal fluid, cat. D. Same cat: Fine to coarse granules in neoplastic granular lymphocytes, aspirate of intestinal lymphoma. Cytocentrifuge (A, B) or direct (C, D) preparations, Wright stain, 100× objective.

phases occur in fluids, but they are commonly lumped into a macrophage category once the cells have left the vasculature.

b. The cytoplasm of macrophages frequently contains vacuoles, lipid, or degraded cellular material (Figures 19.5B and 19.6A), and might contain engulfed RBCs, organisms, or foreign material (Figure 19.9). In vitro, contaminating RBCs, organisms, or foreign material can undergo phagocytosis within 15–30 min (Figure 19.9C and D).
   (1) Macrophages containing RBCs are relatively common in effusions when blood contaminates a fluid and when cytopreparations are not made soon after sample collection.
   (2) Macrophages containing neutrophils are relatively common in fluids because macrophages remove neutrophils by phagocytosis. Frequently, macrophages contain neutrophils at various stages of degradation (Figure 19.5B).

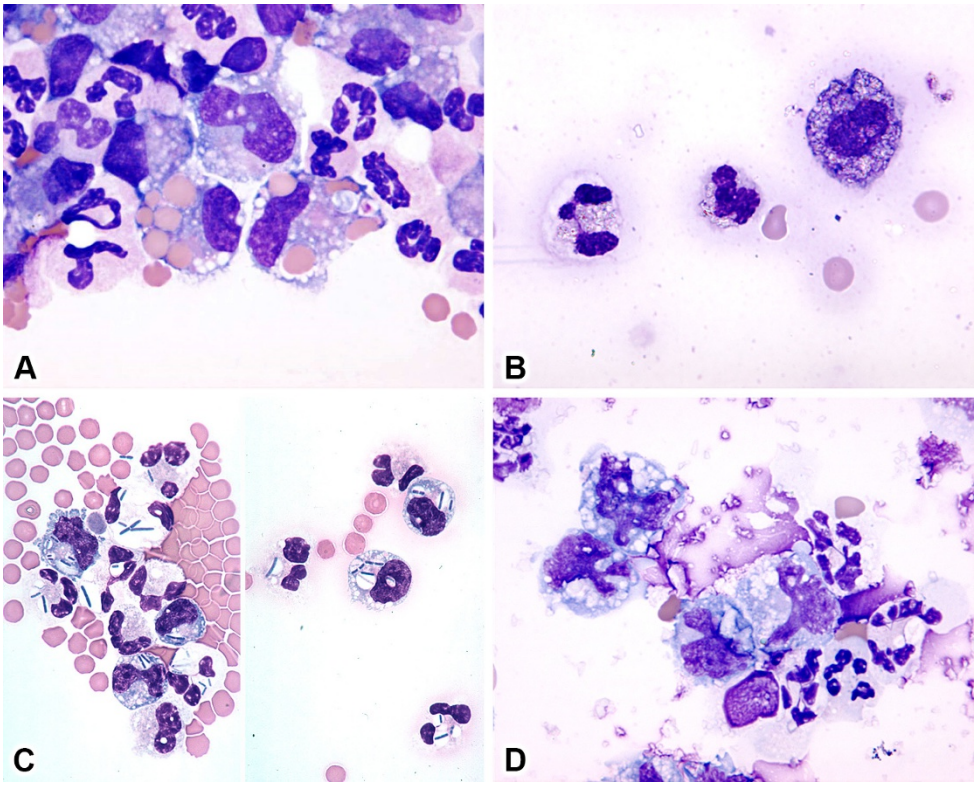


Figure 19.9. Phagocytosis by macrophages. A. Two macrophages containing RBCs (*middle bottom*), pleural fluid, horse. B. A macrophage (*right*) and two neutrophils containing barium – refractile, nonstaining, granular mineral – that escaped through an intestinal defect assessed by a radiographic barium series, peritoneal fluid, cat. C. EDTA-tube sample of peritoneal fluid immediately contaminated in vitro with a bacterial culture broth and processed within 30 min to show in vitro phagocytosis of bacteria by macrophages (bluer cytoplasm) and neutrophils; healthy horse. Note: This type of bacterium is expected only in neutrophils with in vivo phagocytosis. D. Nondegenerate neutrophils and macrophages with irregularly shaped and sized nonstaining intracellular and extracellular silica particles from collecting fluid into an activator-containing clot tube (in vitro phagocytosis), peritoneal transudate, cat. Note: Silica is more variable than barium. Cytocentrifuge (A, C, D) or direct (B) preparations, Wright stain, 100× objective.

4. Mesothelial cells
   a. *Mesothelial cells* are of mesodermal origin and occur as individual cells or sheets of squamous epithelial cells in health. They have round to oval nuclei and usually moderate amounts of pale blue cytoplasm. Individual cells usually are round to oval; cells in sheets can be polyhedral or appear spindloid (Figure 19.10).
   b. *Reactive mesothelial cells*, which are round to oval, have various degrees of increased cytoplasmic basophilia and altered chromatin patterns (Figure 19.10C and D). These proplastic cells may be multinucleate or have mitotic nuclei, and they may have small cytoplasmic vacuoles, prominent glycocalyx fringes, and cytoplasmic blebbing.

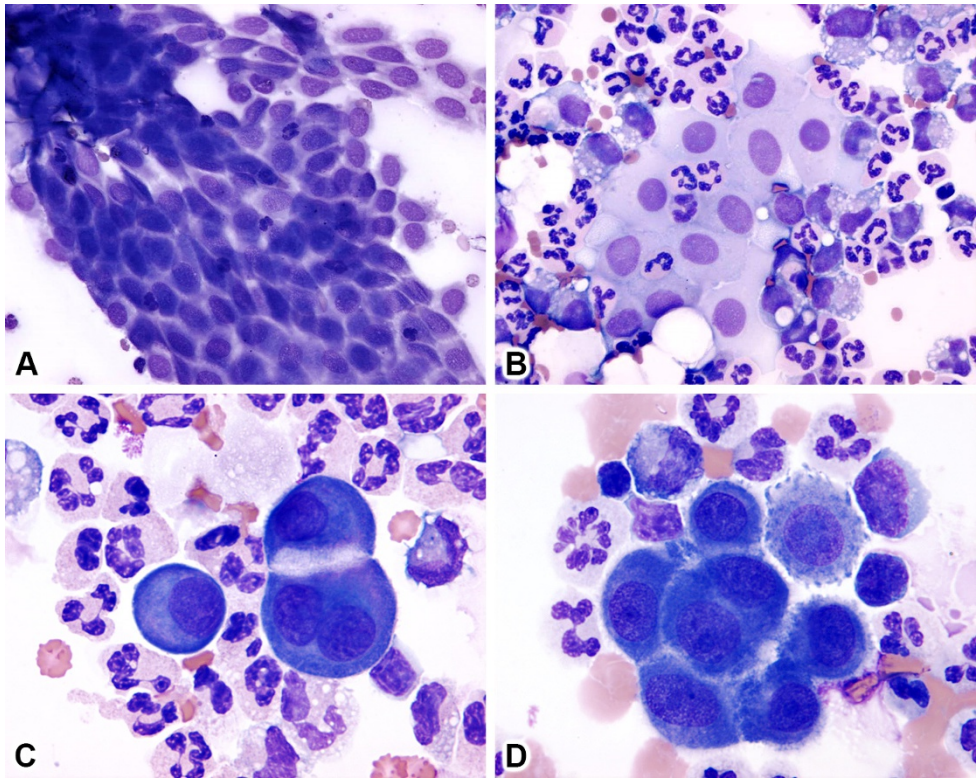


Figure 19.10. Mesothelial cells. A. Sheet of nonreactive mesothelial cells with ovoid nuclei and pale cytoplasm, ox. B. Small sheet of nonreactive mesothelial cells, neutrophils, and macrophages, peritoneal fluid, horse. C. Single and doublet reactive mesothelial cells amidst mostly nondegenerate neutrophils, pleural fluid, dog. Note: Reactive mesothelial cells have increased cytoplasmic basophilia, are commonly binucleate, and may have prominent nucleoli and other features of malignant cells (not shown). D. Central cluster of reactive mesothelial cells, some forming a typical cohesive "ball" and some (*right side*) with marginal cytoplasmic blebbing and brush borders typical of mesothelial cells, pleural fluid, dog. Wright stain, 50× (A, B) and 100× objectives (C, D).

c. Besides their structural functions, mesothelial cells are involved in inflammatory responses of pleural and peritoneal cavities, including antigen presentation, cytokine production, release of oxidants and proteases, promotion of the migration of neutrophils, and tissue repair.[65,66]

5. Mononuclear cells
   a. The term *mononuclear cells* is often used collectively for cells that are not granulocytes or mast cells (though they also have only one nucleus, and macrophages may have multiple nuclei). Most so-called mononuclear cells in effusions are lymphocytes, macrophages, or mesothelial cells, and they can be categorized as such in most cases. In well-prepared, well-stained cytopreparations, most small lymphocytes and macrophages that have vacuolated cytoplasm or contain engulfed material are easily recognized. However, some reactive lymphocytes and smaller monocytoid or histiocytic cells are not easily differentiated. Typically, those cells are a minority part of an inflammatory response, and accurate differentiation is probably not needed.

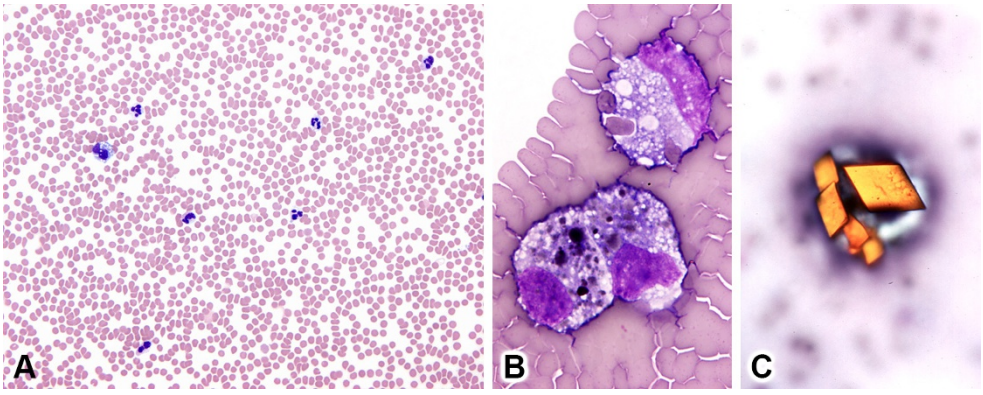


Figure 19.11. Hemorrhagic effusion (pathologic hemorrhage). A. The body of a direct smear looks like blood without platelets, hemorrhagic pericardial effusion, dog, 20× objective. B. Three macrophages containing RBCs or brown to black hemosiderin at the feathered end of a direct smear of pathologic hemorrhage, 100× objective. C. Cluster of hematoidin (bilirubin) crystals in an out-of-focus macrophage, pericardial effusion, dog, 100× objective. Wright stain (hematoidin does not stain, but the protein–iron of hemosiderin stains and can appear grey, green, brownish, or black).

   b. Very degenerate neutrophils may be mistaken for macrophages and therefore so-called mononuclear cells. This is most common with bacterial exudates.
   c. Some histiocytic cells and reactive mesothelial cells have similar nuclear and cytoplasmic features; consistent differentiation may not be possible. Accurate classification is generally unnecessary because these ambiguous cells are usually minor contributors to the TNCC, and differentiation does not alter the overall interpretation of the results.

6. RBCs are found in nearly all samples. Abnormal microscopic features of RBCs often represent artifacts (e.g., membrane spicules, hypochromia, and refractile membranes) but may reflect pathologic states (e.g., acanthocytes, polychromasia, and associated organisms). Findings suspected to represent a pathologic state should be confirmed by finding the same abnormality in a stained film of fresh blood.
   a. If the hemorrhagic effusion has been present for at least a few hours, erythrophages may be found (Figure 19.11B);[67] it may take 2–4 d for hemosiderin to become apparent in macrophages.[67,68] Also, hematoidin (bilirubin) crystals may be found in macrophages (Figure 19.11C).
   b. Macrophages containing RBCs, hemosiderin, or hematoidin crystals become more prominent in effusions the longer the blood has been in a cavity.
   c. When the hemorrhage is caused by a nontraumatic event (e.g., hemorrhagic neoplasm), there is frequently either recurring hemorrhage or persistent low-grade hemorrhage. Thus, the effusion may have properties of both an acute hemorrhagic effusion (i.e., high Hct value) and a chronic hemorrhagic effusion (i.e., hemosiderin within macrophages).
   d. If the making of a cytopreparation was delayed for a few hours, or less in some cases, then the presence of erythrophages may represent in vitro phagocytosis.

D. Platelets can be found in collected samples. The presence of platelets suggests ongoing (or very recent) hemorrhage, or contamination of fluid with blood during collection. Clots indicate fibrinogen was in the sample, but the fibrinogen could be present because of exudation, hemorrhage, or blood contamination.

E. Nucleated cells that are less commonly found in pleural and peritoneal fluids
   1. Eosinophils usually have features similar to those seen in blood (Figure 19.12A). They rarely predominate. Increases suggest parasitic disease (e.g., dirofilariasis, lungworms), hypersensitivity disorders (e.g., allergic conditions, reaction to foreign material), neoplasms (e.g., lymphoma, mast cell neoplasm, carcinomas), and rarely fungal infections (e.g., coccidioidomycosis).
   2. The presence of relatively few mast cells (Figure 19.12B) is not unusual in exudates and also occurs with other effusions (e.g., cardiac failure effusions). Unless the cells have prominent microscopic features of malignancy, neoplastic and nonneoplastic mast cells cannot be reliably differentiated via microscopy.
   3. Neoplastic cells are not common in effusions (Figures 19.8, 19.13, and 19.14). More information on neoplastic cells is found in Comments about Specific Effusions, Section V.

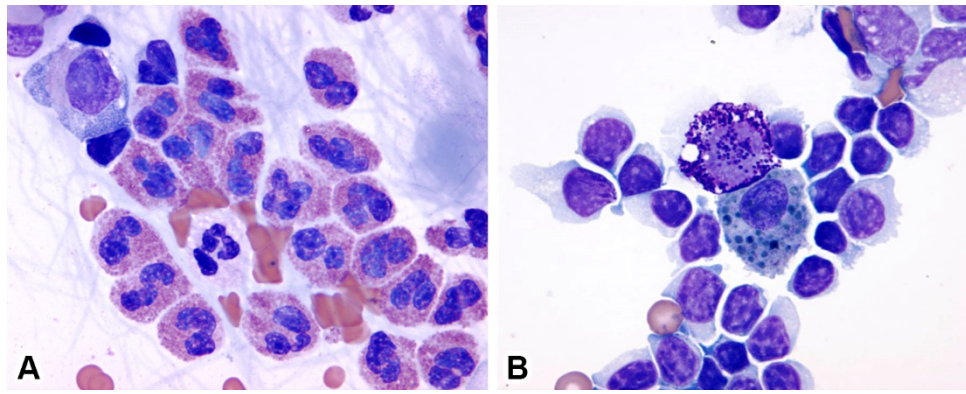


Figure 19.12. Eosinophils and mast cells. A. Mostly eosinophils and blue fibrinous material supportive of fibrin formation in a rare eosinophilic and proteinaceous exudate, cat. B. A well-granulated mast cell above a macrophage containing hemosiderin and accompanied mostly by nonneoplastic lymphocytes, pleural effusion, dog. Wright stain, 100× objective.

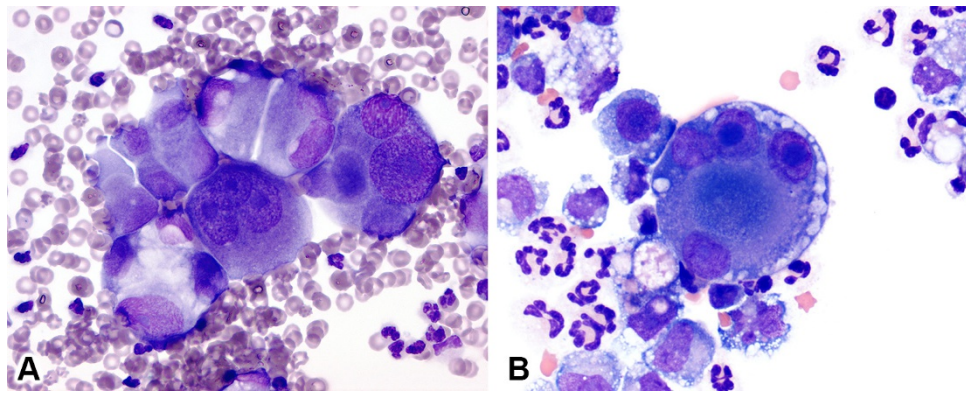


Figure 19.13. Carcinomatous effusions. A. Cluster of pleomorphic large cells with hemorrhage and neutrophilic inflammation, metastatic mammary gland carcinoma, direct smear of pleural fluid, dog. B. Mostly nondegenerate neutrophils and macrophages with two adjacent atypical cells, one being a large multinucleate cell staring up with pupil-like nucleoli bigger than RBCs, carcinomatous pleural effusion, cytocentrifuge preparation, cat. Wright stain, 50× objective.

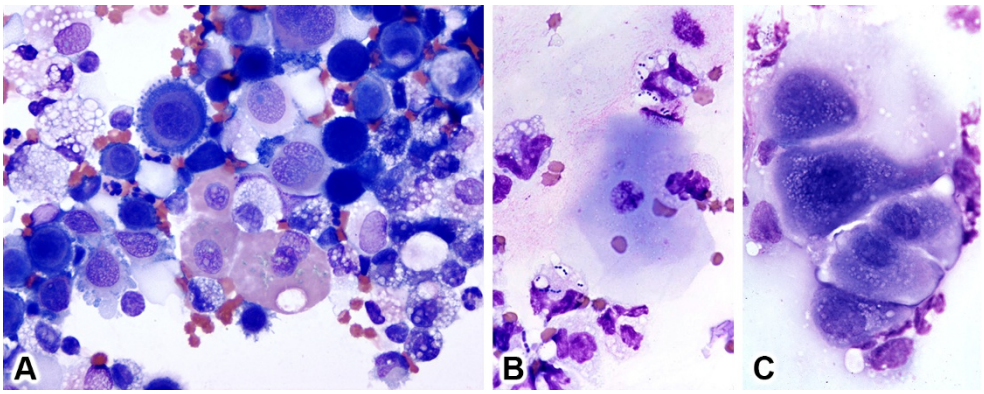


Figure 19.14. Hepatocytes and squamous cells. A. Cluster of three pinkish-tan hepatocytes, one binucleate and two containing green lipofuscin, amidst mostly macrophages and reactive mesothelial cells in a proteinaceous effusion from an attempted thoracocentesis with liver contamination from the abdominal cavity, cat. B. Angular, light-blue superficial squamous epithelial cell surrounded by degenerate neutrophils, some containing short chains of cocci, low-cellularity peritoneal effusion caused by gastric rupture and septic inflammation, horse. C. Cluster of atypical squamous epithelial cells with fine perinuclear vacuolation, metastatic squamous cell carcinoma, pleural fluid, horse. Cytocentrifuge preparations (A, B) and direct smear (C), Wright stain, 50× (A) and 100× objectives (B, C).

4. Occasionally, cells from tissues are found in effusions. Hepatocytes are occasionally found as contaminants in pleural and peritoneal fluids, although this was more common prior to ultrasound-guided collection methods (Figure 19.14A). Squamous epithelial cells from skin may enter the fluid during the collection process, but they may also be present in equine peritoneal effusions because of gastric rupture (Figure 19.14B), in equine peritoneal or pleural fluid because of squamous cell carcinoma (Figure 19.14C), or they may be in pleural fluid in association with esophageal rupture.

F. Noncellular findings
   1. Organisms (primarily bacteria and fungi) can be found in exudates.
      a. Most undegraded bacteria stain dark blue (typically bluer than nuclei), dark purple, or dark grey with a Romanowsky stain (see Chapter 2), and usually can be identified as cocci or bacilli (Figure 19.15A), though other forms exist. They may be paler and pinkish or purplish when partially degraded.
      b. *Mycobacterium* spp. do not stain with a Romanowsky stain and therefore appear as nonstaining (white) bacilli, commonly in macrophages and also in neutrophils.
      c. *Actinomyces* and *Nocardia* may not be uniformly distributed in an exudate. Squash preparation of yellow particulates (*sulfur granules*) in an exudate may reveal a colony of filamentous, beaded, branching bacilli consistent with such bacteria (Figures 19.6B and 19.15B). *Fusobacterium* bacteria may also be beaded and filamentous but do not branch.
      d. WBCs containing anaplasmal morulae (e.g., *Anaplasma phagocytophilum, Ehrlichia ewingii*) may be found in effusions; they represent blood cells that migrated into the fluid (Figure 19.15C).
      e. Dead or damaged bacteria may stain a variety of colors from red to light grey or light purple. The shapes of the dead organisms may be different from those that were live when the sample was prepared for examination.

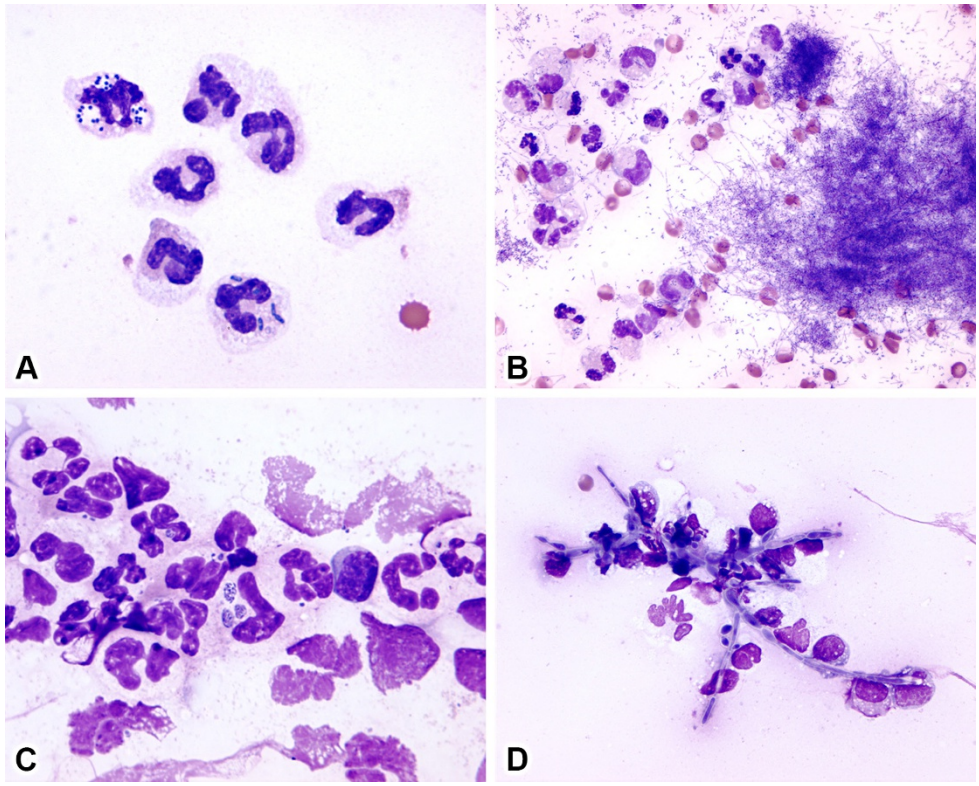


Figure 19.15. Bacterial and fungal effusions. A. Neutrophilic inflammation with phagocytosis of bacterial cocci (*upper left*) and bacilli (*bottom*) three days after intestinal resection and anastomosis, peritoneal exudate with positive culture for *Enterococcus* and β hemolytic *Streptococcus*, dog. B. Neutrophilic inflammation associated with a mat of bacteria including beaded filamentous forms supportive of *Actinomyces* or *Nocardia*, pyothorax, dog. C. Feathered end of smear with mostly neutrophils, one containing three morulae of *Anaplasma phagocytophilum*, peritoneal exudate, horse (ASVCP slide contributed by D. Wood et al., 2001). D. Pseudohyphae of *Candida* sp. with damaged adherent macrophages and neutrophils; pericardial mycotic exudate, immunosuppressed dog. Direct smears, Wright stain, 100× (A, C) and 50× (B, D) objectives.

f. Fungi may appear as yeast (e.g., *Blastomyces* or *Histoplasma*), hyphae (e.g., *Aspergillus* or *Mucor*), or yeast and pseudohyphae (*Candida*) (Figure 19.15D). Most yeast stain blue to purple with Wright stains, but hyphae may have negative reactions; the walls of both forms may be nonstaining. Nonstaining hyphae can be detected as delineated linear structures that are displacing cells and other stained material.

g. Larvae (tetrathyridia) of *Mesocestoides* sp. may be found in dogs with peritoneal[69] or, rarely, pleural cestodiasis.[70] The larvae develop in intermediate hosts prior to ingestion, and while some develop into mature intestinal adult tapeworms, others may migrate from the intestine to body cavities where they proliferate asexually.

h. Tachyzoities or merozoites of apicomplexan protozoa can occasionally be found in exudates: *Neospora caninum* in canine peritoneal fluid[71] and *Toxoplasma gondii* in feline pleural[72] and peritoneal fluids.[73]

i. Large ciliated protozoa (e.g., *Balantidium* sp., *Polymorphella* sp., and *Cycloposthium* sp.) may be found in equine peritoneal exudates or in intestinal taps (Figure 19.16)[74]

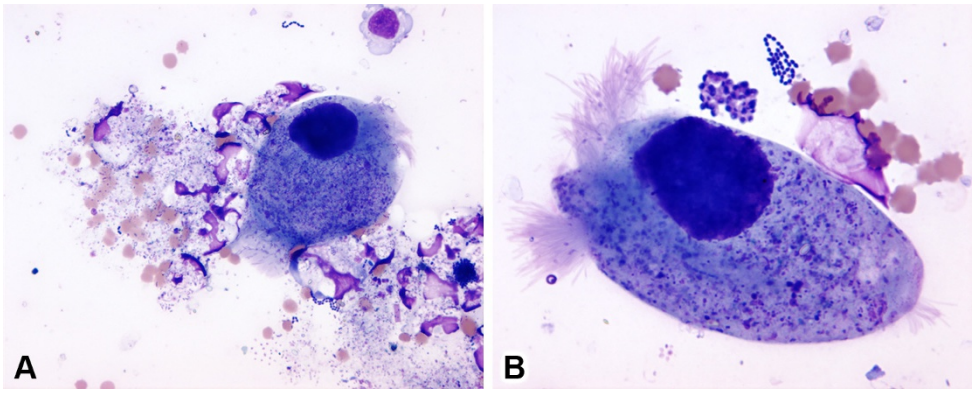


Figure 19.16. Protozoa from ruptured equine intestine. A. Ciliated intestinal protozoon and mostly neutrophils with nuclei distorted by intracellular bacteria and other material, also present extracellularly; low-cellularity peritoneal fluid with septic neutrophilic inflammation, 50× objective. B. Same fluid with a similar protozoon and clusters of extracellular bacteria (plus a face! ☺) 100× objective. Cytocentrifuge preparation, Wright stain.

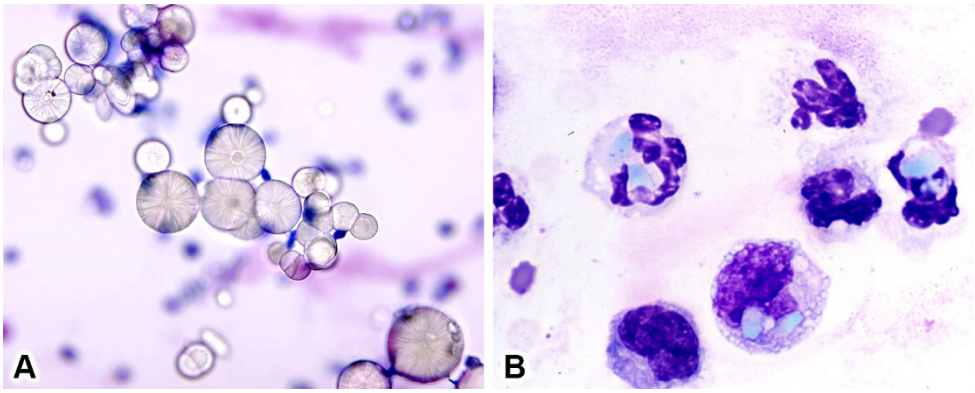


Figure 19.17. Urine crystals and sperm. A. Calcium carbonate crystals and out-of-focus nucleated cells, uroperitoneum, horse, 20× objective (ASVCP slide contributed by L. Vap et al., 1994). B. Pale-blue sperm heads in neutrophils and a macrophage, seminoperitoneum, mare, 100× objective (ASVCP slide contributed by P. McWilliams, 1992). Wright stains.

The protozoa typically are commensal inhabitants of equine intestines but may be secondary invaders of diseased intestinal mucosa.

j. Microfilaria (e.g., *Dirofilaria immitis* or *repens* in dogs, *Setaria* spp. in horses) may be seen in hemorrhagic or nonhemorrhagic effusions, but they are rare and generally incidental to the cause of the effusion. When present, they should prompt investigation and appropriate treatment for previously unrecognized parasitism.

2. Other material that can be found in exudates and lipid effusions includes urine crystals (Figure 19.17A), sperm (Figure 19.17B), bilirubin crystals (hematoidin, Figure 19.11C), bile (Figure 19.18A), mucus (Figure 19.18B), fibrin (Figure 19.18D), plant material, barium crystals (Figure 19.9B), and lipid droplets, including mineral oil. Sudanophilic droplets can be identified in the fluid and in macrophages by using a Sudan stain. To look for Sudanophilic droplets, Sudan stain is placed on top of an

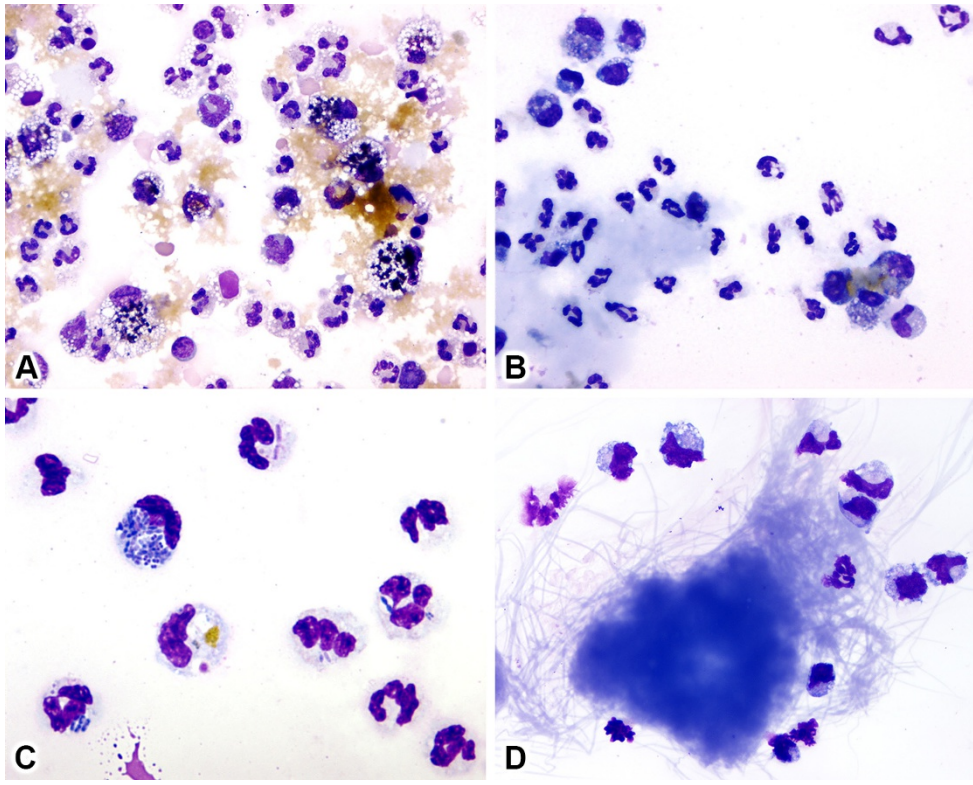


Figure 19.18. Bile, mucus, fecal pigment, and fibrin in effusions. A. Neutrophils and macrophages with intracellular and extracellular amorphous bile pigment, bile peritonitis, dog, 50× objective. B. Bile pigment (*lower right*) and amorphous mucoproteinaceous material (pale blue, likely biliary) with macrophages and neutrophils (distorted), peritoneal exudate in a dog with mucinous hyperplasia and focal necrosis of the gallbladder, 50× objective. C. Neutrophils containing mixed bacteria and fecal material with a brownish yellow color from bilirubin or a derivative, septic peritonitis from intestinal perforation, dog, 100× objective. D. Fibrin clot, similar to mucus or nonfibrin protein (not shown) but with fibrinous strands, proteinacious peritoneal effusion, cat, 50× objective. Wright stains.

air-dried cytopreparation of the effusion, and then a coverslip is placed on top of the liquid. Via microscopy, the Sudanophilic droplets appear as round, orange to red structures that tend to float (just under the coverslip) or are within macrophages.

3. The staining intensity of the space between cells tends to be greater when the fluid has a high [TP] (more protein residue). A drying artifact may create protein crescents when the [TP] is increased (Figure 19.19).

SELECTED ANALYSES FOR PLEURAL AND PERITONEAL EFFUSIONS

I. [Cholesterol] and [TG]
   A. If a diagnostician suspects an effusion is chylous but routine analysis does not provide enough evidence for a firm conclusion, measuring the [cholesterol] and [TG] of the effusion and the animal's serum may provide data to differentiate chylous and nonchylous effusions.

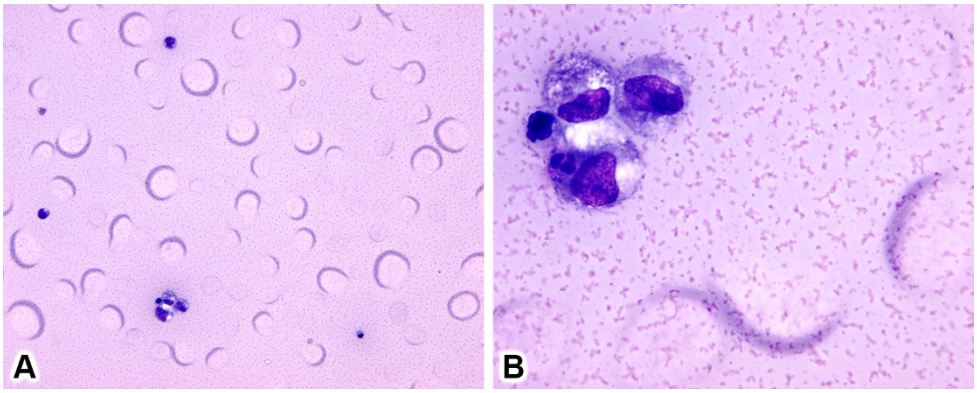


Figure 19.19. Protein crescents and stippling. Lower and higher magnifications of protein crescents and stippled protein residue with few macrophages, proteinaceous exudate of FIP, cat. Direct smear, Wright stain, 20× (A) and 100× (B) objectives.

Confirmation of a chylous effusion indicates that there is a lesion involving lymphatics that drain the intestine, but it does not indicate the cause.

1. Typically, the [TG] in a chylous effusion is much greater than the animal's serum [TG], but reported feline data show they may be similar in TG-poor chylous effusions (see feline [TG] in Table 19.7). A Chol:TG ratio (see below) may be needed to differentiate chylous from nonchylous effusions. A fluid to serum [TG] ratio < 1 does not support a chylous effusion.
2. The [TG] in a chylous effusion is expected to be greater that serum [TG] because chylomicrons are TG-rich molecules; comparing the effusion concentrations via a TG:Chol ratio (Table 19.7) emphasizes that chylomicrons are relatively TG-rich and cholesterol-poor molecules. Even if an animal has hypertriglyceridemia due to a postprandial hyperlipemia or a pathologic state, the chylomicrons are too large to pass across normal blood or lymphatic vessels. Chylomicrons contain relatively little cholesterol and thus the cholesterol concentrations in the chylous effusion may be similar to the patient's serum [cholesterol] (Table 19.7).
3. In human medicine, the cholesterol content of pleural effusions (not peritoneal) has been used to help differentiate transudates from exudates;[40] increased vascular permeability of inflammation may allow cholesterol-containing lipoprotein molecules to enter pleural exudates. The effusion [cholesterol] was low (4–31 mg/dL) in 14 dogs with pleural or peritoneal effusions attributed to chronic renal disease, acquired portosystemic shunts, or protein-losing enteropathy (low-protein transudates) and higher (41–251 mg/dL) in 27 dogs with pleural or peritoneal effusions attributed to cardiac disease, caudal vena cava syndrome, or neoplasms.[77]

B. In a study involving dogs and cats,[75] effusions were considered to be chylous if (i) there was microscopic evidence of chylomicrons (Sudanophilic droplets), (ii) the sample could not be cleared with centrifugation, or (iii) the fluid did clear after the addition of ether. Effusions were classified as nonchylous if they did not meet the chylous effusion criteria. The results of the study are in Table 19.7.
   1. As would be expected with the presence of Sudanophilic droplets in the chylous effusions but not in the nonchylous effusions, the triglyceride concentrations were greater in

Table 19.7. The [TG], [cholesterol], and Chol:TG ratios in chylous and nonchylous pleural effusions of dogs and cats

		Dogs		Cats	
		Chylous	Nonchylous	Chylous	Nonchylous
Fossum study[75]					
Number in group		6	3	4	5
Cholesterol (mg/dL)	Effusion	37–127	48–139	38–232	65–111
	Serum	102–225	127–237	130–202	129–150
Triglyceride (mg/dL)	Effusion	445–2687	8–33	91–4670	0–59
	Serum	21–35	23–45	89–355	43–190
Chol:TG ratio	Effusion	0.04–0.16	3.88–6.00	0.05–0.70	1.88–110.00
TG:Chol ratio[a]	Effusion	6.3–25.0	0.2–0.3	1.4–20.0	< 0.1–0.5
Waddle study[76]					
Number in group		6	17	13	12
Cholesterol (mg/dL)	Effusion	23–70	3–119	18–148	16–107
Triglyceride (mg/dL)	Effusion	110–610	3–83	130–3490	12–93
Chol:TG ratio	Effusion	0.04–0.64	0.33–27.33	0.01–0.64	0.23–7.64
TG:Chol ratio[a]	Effusion	1.6–25.0	< 0.1–3.0	1.6–100.0	0.1–4.3

[a] TG:Chol ratios were calculated from the reported Chol:TG ratios in each study; values were rounded to the nearest 0.1.

the chylous than in the nonchylous effusions. Also, the Chol:TG ratios were much smaller (TG:Chol ratios were much larger) in the chylous effusions because of the greater triglyceride concentrations (Table 19.7).

2. The diagnoses for dogs with chylous effusions included thoracic lymphangiectasia, intestinal lymphangiectasia, and aortic body neoplasm.
3. The diagnoses for cats with chylous effusions included thoracic lymphangiectasia, diaphragmatic hernia, cardiomyopathy, and metastatic pulmonary adenocarcinoma.

C. In another study involving dogs and cats,[76] effusions were considered to be chylous effusions if lipoprotein electrophoresis revealed a distinct chylomicron band at the origin of the electrophoretic pattern.

1. The major findings in this study's laboratory data were as follows (Table 19.7):
   a. Each chylous effusion had a [TG] >100 mg/dL, and each nonchylous effusion had a [TG] < 100 mg/dL.
   b. Each chylous effusion had a Chol:TG ratio < 1, whereas most nonchylous effusions had Chol:TG ratios > 1. As shown in Table 19.7, there was overlap in the Chol:TG ratios (and TG:Chol ratios) in both dogs and cats.
   c. Grossly, nearly all chylous effusions had white, milky appearances after centrifugation, whereas none of the nonchylous effusions had that appearance.
2. The diagnoses for dogs with chylous effusion included lymphoma, venous thrombosis, cardiomyopathy, and idiopathic chylothorax.
3. The diagnoses for cats with chylous effusion included cardiomyopathy, diaphragmatic hernia, pericardial effusion, and idiopathic chylothorax.

D. A pancreatitis-associated effusion in a dog or cat may have a [TG] > 100 mg/dL and a Chol:TG ratio < 1, in some cases with postcentrifugation turbidity.[77] However, in such reported cases, chylomicrons were absent based on lipoprotein electrophoresis, and effusions lacked the prominence of lymphocytes expected for a chylous effusion.

E. Cholesterol and TG assays that are designed for serum samples (see Chapter 16) typically can be used to analyze the supernatant of suspected chylous effusions. When the sample looks like milk, the sample may need to be diluted before the [TG] is measured.

II. [Urea] and [Creatinine]

A. When a diagnosis of uroperitoneum is being considered, the measurement of [Crt] or [urea] (may be reported as [urea nitrogen]) in the peritoneal fluid and a time-matched serum, plasma, or whole blood sample may help establish a diagnosis. It is important that the effusion concentrations be compared with concurrent or very recent serum concentrations because of the rapid diffusion of Crt and urea from the cavitary fluid to lymph and blood.

B. Urea and Crt are small molecules that diffuse through capillary walls, thus their concentrations in blood and extravascular fluids equilibrate with time. In health and most pathologic states that create effusions, the serum and peritoneal fluid concentrations of urea and Crt are nearly the same.

C. When there is a recent addition of urine to peritoneal fluid, the resulting fluid has greater urea and Crt concentrations than plasma concentrations because the [urea] and [Crt] in urine are typically much greater than plasma or serum concentrations. With time, lymphatic drainage, and diffusion of molecules due to concentration gradients, their concentrations in plasma will increase and become similar to those in the peritoneal fluid.

1. After 45 h of experimentally induced uroperitoneum, the [Crt] in peritoneal fluid was about twice the serum [Crt]: The mean peritoneal fluid [Crt] of nonsurvivors was 11.6 mg/dL (range, 5.5–15.6 mg/dL), whereas the mean serum [Crt] was 5.2 mg/dL (range, 1.2–6.7 mg/dL). However, the [urea] in the effusion and serum at 45 h were nearly the same. These data support the fact that urea enters the blood faster than does Crt. Azotemia (increased [urea] or [Crt]) was detectable in a few dogs within 5 h but in all 11 dogs by 21 h after "rupture." The serum [urea] and [Crt] continued to increase over the 3-d study.[78]
2. In a retrospective study of 13 dogs with uroperitoneum, the Crt concentrations in peritoneal fluid were at least twice the "blood" concentrations in 11 dogs. Durations of the spontaneous conditions were not known.[79] The ratios for eight dogs with other peritoneal effusions were from 0.7 to 1.2.
3. Ratios of peritoneal fluid [Crt] to serum [Crt] have been reported for other animals with uroperitoneum: foals (mean, 3.5; range, 2.7–4.6),[80] horses (> 2.0),[81] and cats (mean, 2.0 and range, 0.8–2.4; samples for the 0.8 ratio may not have been collected at the same time).[82]
4. The ratios can be used as a guideline in the interpretation of data, but other case information should be considered. For example, a cat with a urinary tract obstruction may have been severely azotemic before the uroperitoneum occurred. Thus, the ratio may not be > 2 when the cat is first examined. No single value can be used alone to completely discriminate uroperitoneum from other effusions based on the ratio of fluid and serum [Crt].

D. Urea (or urea nitrogen) and Crt assays that are designed for serum samples (see Chapter 8) typically can be used to analyze the supernatant of suspected uroperitoneal effusions. For some assays, the sample may need to be diluted before analysis to obtain analyte concentrations within the assay's analytical range.

III. [Na^+], [Cl^-], and [K^+]

A. Na^+, Cl^-, and K^+ are freely diffusible through most capillary walls, and thus their concentrations in plasma, interstitial fluids, and most pleural and peritoneal effusions are nearly the same. The major exception to this concept occurs in uroperitoneum. Compared to plasma and most extracellular fluids, the urine [Na^+] and [Cl^-] are less and the urine [K^+] is greater. Thus, in early stages of uroperitoneum, the peritoneal fluid [Na^+] and [Cl^-] are less than plasma concentrations, and the peritoneal fluid [K^+] is greater than plasma concentrations. With time, diffusion of electrolytes and H_2O reduce the differences.

B. In a retrospective study of 26 cases of feline uroperitoneum, the [K^+] was measured in five cats. The ratios of the peritoneal fluid [K^+] to serum [K^+] ranged from 0.9 to 2.4, with a mean of 1.2.[82]

C. In a retrospective study of 13 dogs with uroperitoneum, ratios of the peritoneal fluid [K^+] to the "blood" [K^+] were ≥ 1.4 in all dogs (median, 2.7; and range, 1.4–4.9). (Note: The authors did not specify whether the "blood" [K^+] was a serum or plasma concentration; serum [K^+] is typically > plasma [K^+].)[79] The ratios for eight dogs with other peritoneal effusions ranged from 0.7 to 1.2.

D. In an experimental production of uroperitoneum in dogs, hyponatremia and hypochloremia were apparent between days 1 and 2 and pronounced by day 4. Hyperkalemia was apparent in some dogs by day 2 and in nearly all dogs by day 4.[78]

E. Assays designed for serum [Na^+], [K^+], and [Cl^-] (see Chapter 9) may not provide accurate electrolyte concentrations in the supernatant of cavitary effusions. Some ion-selective electrode methods require a proteinaceous fluid, so urine or a uroperitoneal effusion may not be acceptable. Anions other than Cl^- may react with the electrodes of the Cl^- assay. For cellular samples, measured [K^+] might include K^+ released from cells either in vivo or in vitro.

IV. [L-Lactate]

A. L-Lactate is the product of anaerobic respiration, and one reason for hyperlactatemia is tissue hypoxia (see Chapter 9). The L-lactate produced by cells may enter venous blood to cause hyperlactatemia, but also may enter interstitial fluids and diffuse into pleural or peritoneal fluids. An increased [L-lactate] in pleural and peritoneal fluids may be a marker of increased anaerobic respiration in the body cavities involving RBCs, WBCs, hypoxic tissues, neoplasms, bacteria, or fungi.

B. An increased [L-lactate] in peritoneal fluids has been found in animals with strangulated and nonstrangulated intestinal obstructions, intra-abdominal neoplasms, and bacterial exudates.

1. The [L-lactate] in peritoneal fluids was greater in colicky horses with intestinal ischemia secondary to strangulating obstruction (8.5 ± 5.5 mmol/L, mean ± standard deviation) compared to colicky horses with nonstrangulating obstruction (2.1 ± 2.1 mmol/L) and healthy horses (0.6 ± 0.2 mmol/L).[83] The [L-lactate] in equine peritoneal fluid is used as an indicator of the severity of intestinal ischemia in colicky horses.
2. The [L-lactate] in peritoneal effusions was greater in dogs with intra-abdominal neoplasms (hemangiosarcoma, carcinomatosis, neuroendocrine neoplasm, metastatic adenocarcinoma, and pancreatic carcinoma) (3.8 ± 1.7 mmol/L, mean ± standard deviation) than in dogs that did not have intra-abdominal neoplasms (1.7 ± 0.5 mmol/L).[84] The greater [L-lactate] in the neoplastic group could be due to increased anaerobic respiration in hypoxic tissue, perhaps because a neoplasm is impairing blood flow to tissue, or to increased aerobic glycolysis of neoplastic cells that are not hypoxic (the Warburg effect).[85]

3. The [L-lactate] in peritoneal effusions is often greater in dogs and cats with bacterial exudates than in dogs and cats with nonbacterial peritoneal effusions caused by a variety of disorders.[86–88]
   a. In the dogs of one study, the [L-lactate] was 4.2 (3.8–8.4) mmol/L (median and range) in the bacterial exudates (n = 3) and 1.9 (1.1–5.7) mmol/L (median and range) in the nonbacterial effusions (n = 4).[86] Similar data were found in another 8 dogs with bacterial exudates and in 11 dogs with nonbacterial effusions.[87] In a study of 18 dogs with bacterial peritonitis and 19 dogs with nonseptic peritoneal effusions, the [L-lactate] in septic effusions was greater than serum [L-lactate] by a median of 4.9 mmol/L, whereas it was a median of 0.2 mmol/L lower than the serum [L-lactate] in the nonseptic effusions.[88] The [L-lactate] had moderate diagnostic accuracy for sepsis. Fluid cell concentrations were not provided to assess their potential contributions to L-lactate concentrations.
   b. In cats of one study, the [L-lactate] was 6.2 (1.3–10.6) mmol/L (median and range) in the bacterial exudates (n = 5) and 1.4 (1.2–1.6) mmol/L (median and range) in the nonbacterial effusions (n = 2).[86] Similar data were found in another nine cats with bacterial exudates, but a wider range of values was found in nine other cats with nonbacterial effusions.[87]
   c. In these studies, the differences between peritoneal fluid [L-lactate] and plasma [L-lactate] were usually greater in the bacterial exudate groups than in the nonbacterial groups. A difference greater than 2 mmol/L was suggested as an indicator of septic peritonitis,[86] but the same criterion would have incorrectly classified three of eight dogs and five of nine cats in the second study.[87] The pathogenesis of the increased [L-lactate] in the bacterial exudates was not explained, but at least two explanations are possible:
      (1) Bacteria can produce both L-lactate and D-lactate, and thus bacteria could be a source of the L-lactate in the bacterial exudates.
      (2) L-Lactate could also be produced by WBCs and RBCs in the exudate. Cell concentrations in the bacterial effusions tended to be greater than those in the nonbacterial effusions.
4. The [L-lactate] in peritoneal fluid was increased within 2 h of experimental intestinal strangulation in dogs, and it continued to increase during the 24-h experiment. The plasma [L-lactate] changed little in the survivor group but increased rapidly after 8 h in the nonsurvivor group.[89]

C. Postsurgical collection of fluid from a closed-suction drain may not provide reliable evidence of a bacterial peritonitis because the altered L-lactate concentrations may reflect changes within the drainage system and not in the peritoneal fluid.[90]

D. Assays designed for serum or plasma [L-lactate] (see Chapter 9) typically can be used to analyze the supernatant of effusions. When interpreting effusion [L-lactate], one should consider that RBCs and WBCs are a potential source of L-lactate. Also, L-lactate could be produced by cells after sample collection, so samples are best processed and analyzed promptly. If this is not possible, samples could be placed in sodium fluoride tubes (see Chapter 9), or fluid could be removed from cells after centrifugation.

V. [D-Lactate]

A. D-Lactate is a stereoisomer of L-lactate (see Molecule 9.2) and is a product of fermentation by some bacteria, including bacteria found in the intestinal tract.

B. In a study of 90 colicky horses, the D-lactate concentrations in deproteinized peritoneal fluids were much higher in horses with septic peritonitis (median 455 μmol/L) and

gastrointestinal rupture (median 600 μmol/L) than in horses with nonstrangulated intestinal obstructions (median 78 μmol/L) and healthy horses (median 39 μmol/L).[91] The enzymatic assay kit was developed for food products (wine, beer, milk, others), and its validation for the study's sample type and processing was not mentioned. Effusive fluid needs to be deproteinized prior to analysis for [D-lactate]; greater D-lactate concentrations were found in nondeproteinized samples when compared to protein-free samples using an enzymatic assay.[92]

VI. [Glucose]

A. Because glucose in a peritoneal effusion comes from plasma, it is important to interpret the [glucose] in effusions with knowledge of the plasma, serum, or whole blood [glucose]. If the same analyzer is not used for both samples, a clinician should know how glucose results for the two analyzers compare.

B. The glucose concentrations in peritoneal effusions were less in dogs and cats with bacterial exudates than in dogs and cats that had nonbacterial peritoneal effusions caused by a variety of disorders; however, the values overlapped and were not statistically different.[86]

1. In dogs, the glucose concentrations were 57 mg/dL (median) in the bacterial exudates (n = 7) and 137 (51–322) mg/dL (median and range) in the nonbacterial effusions (n = 11).
2. In cats, the glucose concentrations were 100 (39–234) mg/dL (median and range) in the bacterial exudates (n = 7) and 141 (107–224) mg/dL (median and range) in the nonbacterial effusions (n = 5).
3. The glucose concentrations in bacterial exudates were less than the corresponding blood glucose concentrations in all dogs and nearly all cats. The authors suggested that a difference of 20 mg/dL or more indicates a bacterial exudate. However, the TNCCs in all nonbacterial effusions were < 13.0 × 10^3/μL, and nearly all bacterial exudates had greater TNCC values. Thus, the differences in [glucose] may have been due to consumption of glucose by nucleated cells and not due to the presence of bacteria.

C. In horses, the glucose concentrations in equine peritoneal exudates with evidence of bacterial sepsis were statistically significantly lower than the glucose concentrations in transudative or proteinaceous peritoneal fluids lacking evidence of sepsis, but there was substantial overlap.[93] The ratio of the plasma [glucose] to the peritoneal [glucose] was > 1.0 in 11 of the 13 horses diagnosed with septic peritonitis and in only 4 of 24 of the other horses. TNCCs were substantially greater in the septic group, so inflammatory cells rather than bacteria might have been most responsible for consumption of glucose (bacteria were not seen microscopically in smears of effusions for 8 of the 13 horses).

D. Postsurgical collection of fluid from a closed-suction drain may not provide reliable evidence of a bacterial peritonitis because the altered glucose concentrations may reflect changes within the drainage system and not in the peritoneal fluid.[90]

E. Glucose assays that are designed for serum or plasma samples (see Chapter 14) typically can be used to analyze the supernatant of effusions. Glucose is consumed by cells (RBCs, WBCs and other nucleated cells, and platelets), so samples should be analyzed soon after collection. If this is not possible, samples could be placed in sodium fluoride tubes (see Chapter 14), or fluid could be removed from cells after centrifugation.

VII. [Bilirubin]

A. Peritoneal fluid [bilirubin] is low and similar to serum [bilirubin] in health. Because conjugated bilirubin is diffusible and plasma proteins are present in most nontransudative

effusions, the [bilirubin] in most nontransudative effusions somewhat parallels the serum [bilirubin], whether icterus is present or not. The major exception to this concept is seen with bile peritonitis and the formation of a bilious exudate. When damage to bile ducts or gallbladder results in the leakage of bile into the peritoneal cavity, peritonitis develops, and the resultant exudate has an increased [bilirubin].

B. There are several potential causes of bile entering the peritoneal cavity, including blunt or penetrating trauma, bile duct rupture secondary to manual expression of the gallbladder, necrotizing cholecystitis or cholangitis, cholelithiasis, and neoplasia of biliary tissues.[94]

C. When an effusion develops secondary to bile leakage, results of fluid analysis reveal the presence of an exudate that typically has a [TP_{ref}] > 2.5 g/dL, a TNCC > 5.0 × 10^3/μL, and > 80 % neutrophils.[94]

1. Microscopic examination may detect a small to large amount of intracellular or extracellular amorphous yellow to brown bile pigment, or bilirubin crystals, which may be extracellular or in macrophages.
2. If the bile leakage is secondary to a bacterial infection, the exudate may contain intracellular or extracellular bacteria.
3. Typically, the [bilirubin] in bile-containing effusions is at least twice an animal's serum [bilirubin].[94,95]
4. When a gallbladder lesion enables biliary mucoid secretion (called white bile despite lack of typical bile constituents) to enter the peritoneal cavity, the resultant effusion may contain light basophilic mucinous material when stained with a Romanowsky stain (Figure 19.18B).[95,96] However, pools of amorphous light basophilic material are commonly seen in the peritoneal fluid of patients without biliary disease (authors' experience), and reports of the material in cytopreparations of effusions associated with biliary rupture have not included control samples from other conditions to determine its specificity for biliary disease.

D. Effusions resulting from gallbladder rupture do not always have increased bilirubin concentrations, and the animals are not necessarily icteric or hyperbilirubinemic.[96,97] This is particularly the case for dogs with gallbladder ruptures associated with mucoceles.

E. Results of blood and urine analyses depend on the cause and duration of biliary tract leakage. For example, if the animal had obstructive cholestasis prior to bile leakage, hyperbilirubinemia may have preceded the bile peritonitis. However, with trauma-induced bile leakage, the hyperbilirubinemia occurs after the onset of bile peritonitis. Likewise, an inflammatory leukocytosis may be the result of bacterial cholangitis that causes bile leakage, or an inflammatory leukocytosis may occur after the onset of acute bile peritonitis.

F. Bilirubin assays that are designed for serum or plasma samples (see Chapter 13) typically can be used to analyze the supernatant of effusions. Bilirubin is degraded by ultraviolet light, and thus effusions should be processed to limit such exposure (an insignificant problem with routine processing and fluorescent lighting).

VIII. [Bile acids]

A. Peritoneal fluid [bile acids] is low and similar to serum [bile acids] in health. Because most plasma bile acids are bound to albumin, disorders that allow albumin to enter the peritoneal fluid also allows plasma bile acids to enter. They may also enter with plasma lipoproteins, which also bind bile acids, more so with hypoalbuminemia.[98,99]

B. Assays used to measure plasma or serum bile acid concentrations may be used for cavitary effusions.

C. When there is a bile peritonitis, the peritoneal fluid [bile acids] and [bilirubin] may be greater than the serum concentrations, but this is not always the case for [bilirubin], and the magnitude of the difference in [bile acids] has not been established. In a study of 5 dogs with known gallbladder rupture, the peritoneal effusions' bilirubin concentrations were low (0.1 and 0.4 mg/dL) in 2 dogs, whereas the [bile acids] in one dog's effusion was > 1,000 µmol/L and its serum [bile acids] was 46 µmol/L.[97] The authors suggested that bilirubin was more diffusible than bile acids and thus was removed from the peritoneal effusion sooner. In a different report, the [bile acids] in the peritoneal fluid of a dog with a ruptured gallbladder and septic peritonitis was 18.6 times that of the serum concentration, and the dog was not hyperbilirubinemic.[100]

D. Hydrophobic bile acid anions (e.g., deoxycholate and chenodeoxycholate) bind strongly to proteins and thus they should not be present in peritoneal effusions unless the effusion is protein-rich or they were present in leaked bile.[101]

IX. [Ammonium]

A. The [ammonium] in peritoneal fluid was increased 24 h after experimental intestinal strangulation in dogs, but the plasma [ammonium] changed little.[89] The increase in the [ammonium] in the effusion may be caused by luminal ammonium passing through devitalized intestinal tissue.

B. Ammonium assays that are designed for plasma samples (see Chapter 13) typically can be used to analyze the supernatant of effusions. In vitro factors can alter the [ammonium] in the collected sample (see Chapter 13).

X. Protein concentrations

A. With the possible exception of immunoglobulins, most proteins detected in effusions represent those that leaked through capillary walls, escaped blood vessels because of hemorrhage, or left lymphatics via lymphorrhage. Greater amounts of protein and larger proteins can pass through the more protein-permeable capillaries (e.g., fenestrated capillaries, capillaries in inflamed tissue).

B. Protein electrophoresis

1. Protein electrophoresis of pleural and peritoneal fluid (especially if paired with serum protein electrophoresis) provides information about the permeability of capillary beds. If the [TP] of the fluid is < 2.0 g/dL, the fluid may need to be concentrated before electrophoresis.
2. In FIP exudates, the electrophoretic patterns of a cat's exudative effusion and its serum are similar because the disorder's vasculitis enables plasma proteins to pass relatively nonspecifically into the pleural or peritoneal fluid.

C. In people, a pleural effusion [TP] to serum [TP] ratio > 0.5 is supportive of exudation if the effusion does not contain blood.[102] This criterion is based on chemical (biuret) protein measurement, not refractometric measurements which could be influenced by interferents.

D. In people, a serum albumin–ascites gradient (serum [albumin] – effusion [albumin]) has been used to help differentiate peritoneal effusions caused by portal hypertension (e.g., cirrhosis and congestive heart failure) from effusions caused by malignancies or inflammatory states; the gradient is greater (> 1.1 g/dL) in the portal hypertension disorders than in the other disorders.[40,103] A serum albumin–effusion gradient may also be useful to help differentiate pleural transudates from exudates.[40] Most dogs with transudates caused by hepatic cirrhosis had a serum albumin–ascites gradient > 1.1 g/dL, but so did

about half of the dogs that had effusions associated with neoplasms, pancreatitis, congestive heart failure, protein-losing enteropathy, and other disorders.[104]

E. Concentrations of positive acute-phase proteins (see Chapter 7, Positive Acute-Phase Proteins) have been measured in effusions, but the available data are insufficient to establish the diagnostic value of such concentrations.
   1. Concentrations of α_1-acid glycoprotein are greater in plasma and peritoneal fluid of most cats with FIP than in healthy cats.[105,106] However, increases in [α_1-acid glycoprotein] are expected with other inflammatory disorders, and the diagnostic utility of [α_1-acid glycoprotein] in effusions is not documented.
   2. Peritoneal fluid concentrations of two positive acute-phase proteins (serum amyloid A and haptoglobin) increased with increasing duration of colic in horses with intestinal obstructions, strangulated intestines, and other inflammatory disorders.[107] The concentrations of positive acute-phase proteins also increased in the sera of these horses.

F. TP and albumin assays that are designed for serum or plasma samples (see Chapter 7, Analytical Principles for Total Protein, Albumin, and Globulins) typically can be used to analyze the supernatant of effusions. The [TP] or [albumin] might be below the assays' analytical ranges in some effusions. The bromocresol green assay for [albumin] may not provide an accurate concentration if the dye binds to globulins. Assays for some proteins may need to be species specific.

XI. Enzyme activity

A. Lactate dehydrogenase (LD) activity: LD is a cytoplasmic enzyme that is present in most cells (see Chapter 12). LD activity is expected to be greater in exudates and neoplastic effusions than in transudates because LD can be released from damaged or deteriorating WBCs, neoplastic cells, and mesothelial cells. Fluid LD activity may be increased in exudative or hemorrhagic effusions if serum LD activity is increased from hepatocellular or myocyte damage.
   1. LD activity is a component of Light's criteria for determining if human pleural effusions are transudates or exudates,[108] although not all effusions are one or the other; the pathogenesis of effusions may be multifactorial. Using Light's criteria (now modified from the original), a human pleural effusion is likely to be an exudate if at least one of the following three criteria is met (and a transudate if no criterion is met).[40]
      a. The ratio of the effusion-to-serum or -plasma protein concentrations is > 0.5.
      b. The ratio of the effusion-to-plasma or -serum LD activities is > 0.6.
      c. The effusion LD activity is > 2/3 the upper reference limit for serum or plasma LD activity (was originally stated as an LD activity of 200 U/L, but methods vary).
      d. This approach has shown high diagnostic sensitivity for detecting human pleural exudates, but the diagnostic specificity has been lower (75–80 %), meaning that some nonexudative fluids are misclassified as exudates.
   2. Cats
      a. In a study of feline pleural effusions, the LD activities were greater in all exudates, neoplastic effusions, and chylous effusions than in cardiac failure effusions.[109] Also, the ratio of pleural fluid [TP] to serum [TP] was usually lower in the heart failure effusions than in the other effusions (methods of measuring [TP] were not stated); the study also used other "Light's criteria" that are used to differentiate transudates and exudates in human medicine.
      b. In a small study of feline pleural and peritoneal effusions, effusion LD activity tended to be greatest in infectious effusions (5 from FIP, 1 bacterial), lowest in

nonneoplastic and noninfectious disorders (11 from cardiomyopathy, 1 from hepatic disease), and intermediate for neoplastic effusions (6 with lymphoma, 5 with carcinomas).[110] Concurrent measurement of LD isoenzyme activities provided moderate diagnostic specificity and high diagnostic sensitivity for neoplastic effusions in this population.

3. Dogs: Evaluation of effusion LD activity in combination with serum [TP] correctly identified 25 pleural effusions as transudates and 73 of 75 pleural effusions as nontransudative while also differentiating all transudates associated with decreased colloid oncotic pressure (dogs with protein-losing enteropathy, nephrotic syndrome, or hepatic insufficiency) from all effusions associated with congestive cardiac disease (increased hydraulic pressure gradients).[111] Only those cases with an apparent pathogenesis of the effusion were included, and hemorrhagic and chylous effusions, which can be identified in other ways, were omitted. The nontransudative group comprised 42 neoplastic effusions, 32 bacterial infections (25 pyothorax, 7 pleuropneumonia), and one uncharacterized effusion associated with an esophageal perforation. The two misclassified effusions were neoplastic.
   a. Effusions were classified as transudative if LD activity was ≤ 100 U/L, which was two-thirds of the serum LD upper reference limit. Effusions were classified as being caused by decreased oncotic pressure if the serum [TP] was ≤ 4.0 g/dL.
   b. When two different published sets of numeric cutoffs for effusion TNCC and pleural fluid [TP] were used to classify the effusions as transudates or exudates, the diagnostic accuracies were only 57 and 71 %. Total protein concentrations were measured chemically.

B. Lipase activity or pancreatic lipase immunoreactivity (PLI): In one study, peritoneal fluids from dogs with acute pancreatitis had much greater lipase activity than other peritoneal effusions and were at least double the animal's serum lipase activity.[112] Similar results were obtained in another study that additionally showed increased PLI in effusions from dogs with pancreatitis compared to effusions in dogs without evidence of pancreatitis.[113] Such testing might be helpful given that pancreatitis-associated effusions provide few clues to the underlying problem. Cytologically, macrophages and less often neutrophils may contain small to moderate numbers of relatively uniform lipid-like vacuoles suggestive of inflammation involving fat (Figure 19.20). Grossly, the effusions can have a hazy supernatant after centrifugation, but they otherwise appear to be low-protein transudates, protein-rich effusions, or exudates without specific features.[77] In some cases, additional testing may yield a fluid to serum [TG] ratio > 1, but chylomicrons were not detected by lipoprotein electrophoresis in the few such effusions tested.[77]

C. Amylase and lipase activity
   1. In a study in which pancreatic necrosis was found postmortem in 10 llamas and 1 alpaca, paired serum and peritoneal amylase and lipase activities were available for 7 animals.[114] In 5 of the 7 cases, peritoneal amylase activity was greater than serum amylase activity. In 7 of 7 cases, peritoneal lipase activity was greater than serum lipase activity. The measured activities were not reported, nor were the magnitude of differences between peritoneal fluid and serum values.
   2. In two 5-d-old Appaloosa foals with postmortem diagnoses of pancreatitis (one acute; one subacute to chronic), the amylase and lipase activities in the peritoneal exudates were much greater than same-day serum samples.[115]

D. Alkaline phosphatase activity: In horses with colic or intestinal lesions, ALP activity may increase in the peritoneal fluid predominantly because of increased granulocytic ALP

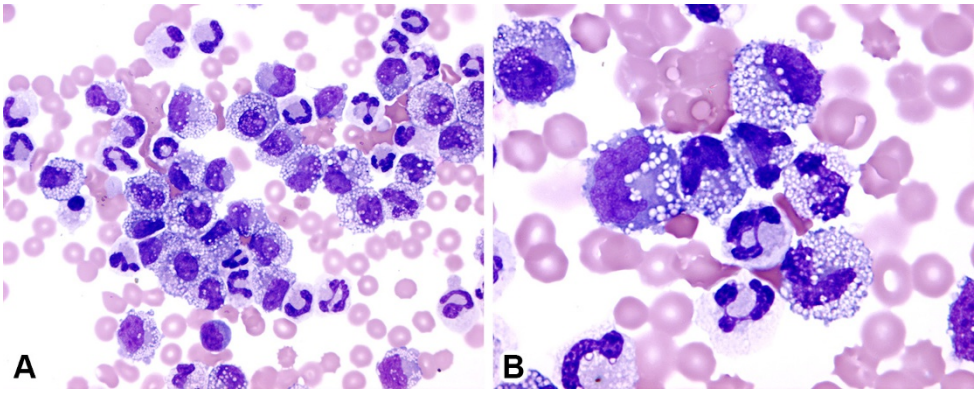


Figure 19.20. Pancreatitis-associated effusion. Macrophages and nondegenerate neutrophils containing many or rare lipid-like vacuoles, respectively, suggestive of inflammation involving fat, mild peritoneal exudate, dog with pancreatitis. Cytocentrifuge preparation, Wright stain, 50× (A) and 100× (B) objectives.

activity,[116] but other data suggest that intestinal ALP may contribute.[117] The magnitude of increased total ALP activity in peritoneal fluid was greater in horses with peritonitis and intestinal rupture than in horses with strangulated and nonstrangulated intestinal disorders; the study's analytical methods did not enable identification of the ALP isoform that produced the increase.[118,119] In these studies, granulocytic and intestinal ALP did not increase total ALP activity in serum.[116,117]

E. Most enzyme assays that are designed for serum or plasma samples (see Chapter 12) typically can be used to analyze the supernatant of effusions.

XII. Amino-terminal proB-type natriuretic peptide (NT-proBNP)

A. NT-proBNP is released from ventricular cardiac myocytes when they are stretched; NT-proBNP is biologically inactive. An increased serum or plasma [NT-proBNP] may be found in animals with a variety of cardiac disorders.

B. NT-proBNP can be measured with a quantitative ELISA or a semiquantitative point-of-care SNAP ELISA using serum or plasma from EDTA-anticoagulated blood. With current tests, serum and plasma samples are best tested fresh or within a few days of refrigeration (no longer than a week); they may also be frozen for later testing.[120] Pleural fluid samples have also been used.

C. A group of 22 cats with various forms of cardiomyopathy (hypertrophic, restrictive, dilated, arrythmogenic, unclassified) had consistently greater pleural [NT-proBNP] (median 1135 pmol/mL) than 18 cats that had noncardiac pleural effusions (neoplastic, chylous, pyothorax, FIP, and pneumonia) (median 52 pmol/mL).[121] The cats with cardiomyopathies also had greater plasma [NT-proBNP] (median 566 pmol/mL) than the noncardiomyopathic cats (median 52 pmol/mL); [NT-proBNP] in plasma and pleural fluid had similar diagnostic value.

D. Other studies of cats with respiratory distress or pleural effusion caused by cardiac or noncardiac disease have shown high diagnostic sensitivity (> 90 %) of pleural fluid and plasma [NT-proBNP] for cardiac disease, but diagnostic specificity was mildly to moderately lower than sensitivity, particularly when using pleural fluid samples rather than plasma.[122] Increased [NT-proBNP] occurred in the plasma or pleural fluid of several cats

with respiratory distress attributed to asthma or neoplasms, and with pleural effusions attributed to other causes (e.g., neoplasms, bacterial infection, FIP). Dilution of pleural fluid prior to analysis may improve diagnostic specificity with the SNAP test.[123]

XIII. Gram staining

A. The Gram stain is used to differentiate bacteria into two large groups: Gram-positive bacteria and Gram-negative bacteria. The Gram stain involves two major steps: (i) staining a bacterium's cell wall with a dye (either methyl violet or crystal violet) and iodine, and (ii) attempting to decolorize the cell wall by using ethanol or acetone solution. In theory, the dye-iodine complex becomes fixed in the Gram-positive organisms and thus they retain the dark purple stain, whereas the dye-iodine complex is dissolved out of the cell walls of the Gram-negative organisms to produce pink organisms.

B. The Gram stain was designed for staining bacteria selected from colonies grown in a variety of culture media, and results of its application to cellular samples or proteinaceous effusions is less uniform and consistent. When the stain is applied to air-dried smears or films of effusions, several factors should be considered:

1. Dead Gram-positive organisms frequently have a Gram-negative reaction; the stain does not remain in the cell walls of dead Gram-positive bacteria during decolorization.
2. Using too little ethanol or acetone may produce a false Gram-positive reaction, whereas using too much ethanol or acetone may produce a false Gram-negative reaction.
3. Cell debris, especially from nuclei, has a Gram-negative reaction, and thus Gram-negative bacteria can be difficult to locate among Gram-negative cellular debris.
4. If used diagnostically, a set of known Gram-positive and Gram-negative slides prepared from cellular effusions should be stained concurrently to serve as positive and negative control slides.
5. The use of Gram stains is not necessary to detect the presence of bacteria. Routine Romanowsky stains are excellent for this and offer much more information, as well.

XIV. Culturing for microorganisms

A. Whenever an infection is suspected, fluid should be submitted for possible culturing of organisms.

B. Usually, culturing can better detect organisms than can a microscopic examination of effusions, especially when very few organisms are in the effusion. However, culture results should be considered in conjunction with microscopic findings, as positive growth may reflect contamination rather than infection, and negative growth sometimes occurs when one or more bacteria are clearly present microscopically.

COMMENTS ABOUT SPECIFIC EFFUSIONS

I. Septic and nonseptic exudates

A. The results of fluid analyses are sometimes classified as either septic exudates or nonseptic exudates.

1. The term *septic* in this context frequently is used synonymously with *bacterial* and is used when bacteria were found during the microscopic examination of an exudate. However, the term *septic* may be applied whenever any microbe is found that is considered the cause of the infectious exudate.

2. The term *nonseptic* in this context frequently is used synonymously with *nonbacterial* and is used when bacteria were not found during the microscopic examination of a stained slide. However, the effusion might be a septic exudate even when bacteria are not found.
   a. Bacteria may have been present but not found via microscopy. This can occur when very few organisms are in a sample.
   b. Bacteria may have been present but not cultured because of inappropriate culture media (e.g., aerobic versus anaerobic organisms) or the animal had been treated with antibiotics prior to collection of an effusion.
   c. The exudation may have been caused by a nonbacterial microbe that was not found by microscopy or culturing.
3. Occasionally, bacteria are found in an exudate but are contaminants. In these cases, the exudate with bacteria might be a nonseptic exudate.

B. A classification of *septic exudate* is best applied to an effusion when it is known that an infectious agent caused or contributed to the exudation. Conversely, a classification of *nonseptic exudate* is best applied to an effusion when it is known that the exudation was not caused by an infectious agent; this is typically not known at the time a fluid sample is evaluated, so the term *nonseptic exudate* is not recommended for the interpretation of fluid analyses. When bacteria are not found during the examination of an exudative effusion, the effusion can be classified as an *exudate of unknown cause* rather than as a *nonseptic exudate*.

II. Exudate of feline infectious peritonitis (FIP)

A. The pathogenesis of the exudation in FIP involves an antibody response associated with viral replication in macrophages and typically is manifested around small and medium-sized veins. The inflammatory response includes monocytes and macrophages with fewer lymphocytes, neutrophils, and plasma cells, creating granulomatous to pyogranulomatous lesions and disrupting vascular basal laminae.[124] Among feline exudates, the effusion tends to be of low cellularity; neutrophils are frequently the predominant cell population, but the exudate can contain a mixture of neutrophils, macrophages, and lymphocytes. The inflammatory reaction increases the permeability of capillaries, thus causing exudation of plasma proteins. Atypical FIP effusions can occur with secondary infections or with lesions that alter lymphatic vessels and cause lymphocytic and sometimes chylous effusions.

B. Several studies have evaluated serum proteins and effusion proteins to attempt to characterize the alterations in protein concentrations. A polyclonal gammopathy with increased [α_2 globulins] is typical but nonspecific for FIP. The electrophoretic protein fractions found in the peritoneal effusions are similar to those found in the cats' sera.[125] Concentrations of α_1-acid glycoprotein are usually increased in a cat's serum and peritoneal effusion.[105,106] Serum or effusion concentrations of haptoglobin, another positive acute-phase protein, may also be increased in cats with FIP, but [haptoglobin] lacks diagnostic utility for FIP.[105]

C. Rivalta's test has some diagnostic value for FIP because it is frequently positive when the exudation is due to FIP.[126–128] However, the Rivalta's test can be positive when the effusion is due to a bacterial peritonitis, lymphoma, or other neoplasms,[126,127] and such disorders must be excluded in order to reach a diagnosis of FIP.
   1. Rivalta's test is described as a simple test in which one drop of acetic acid (98 % or 98–100 %) is added to either 5 mL or 7–8 mL of H_2O to create a dilute acetic acid solution; reported pH values of these solutions were not found, and it has not always been clear if distilled or tap H_2O was used. One drop of effusive fluid is carefully added to the surface of the fluid. A positive result is recorded if a precipitate or turbidity is formed; a negative result is recorded if the solution remains clear.

2. The original purpose of the Rivalta's test was to differentiate transudates (negative result) and exudates (positive result) in human effusions.[129] When described in the late 1800s, F. Rivalta (an Italian physician) prepared the reagent by adding 4 drops of glacial acetic acid (H_2O-free acetic acid) to 200 mL of H_2O. The test involved adding 1 drop of the effusion to 200 mL of reagent, and a positive result was recorded if a whitish or milky contrail fell to the bottom of the reagent.
3. The precipitated proteins of positive Rivalta's tests in human pleural and peritoneal effusions were characterized by immunoblotting, and eight proteins were identified: C-reactive protein, α_1-antitrypsin, α_1-acid glycoprotein, haptoglobin, transferrin, ceruloplasmin, fibrinogen, and hemopexin.[130] Except for transferrin, these proteins are positive acute-phase proteins, and fibrinogen has the largest molecular weight of the group. The authors did not report if they attempted to detect other proteins such as immunoglobulins or albumin. The authors did report that the degree of precipitation varied with the pH of the acetic acid solution (precipitation was best with a pH of 3.8–4.2) and no reaction occurred if the pH was $\geq$ 4.6. They also reported that hydrochloric acid and nitric acid solutions produced the strongest reactions at a pH of 3.6, and thus acidity is the major factor responsible for a positive reaction. Precipitation of proteins by acidifying a solution is isoelectric point precipitation.

D. Similar to the Rivalta test, proteins in high-protein FIP or FIP-like effusions may precipitate in the acid reagent of the BASO channel used to measure nucleated cell concentration by the Sysmex XT-2000iV.[131] Consequently, cells can be trapped with protein precipitates, resulting in a falsely decreased BASO-channel nucleated cell concentration. This results in an increased difference (delta WBC) between the nucleated cell concentrations measured in the analyzer's DIFF and BASO channels (delta [WBC] = DIFF [WBC] – BASO [WBC]), a finding that could suggest consideration of FIP.[131,132]

E. Molecular techniques that assess for mutated feline coronavirus can be applied to effusions, blood, and solid tissues; these may provide support for or against a diagnosis of FIP.[132]

F. The gold standard for the diagnosis of FIP is the detection histologic lesions typical of FIP combined with associated lesional immunohistochemical positivity for feline coronaviral antigens. This is most often achieved postmortem. Antemortem assessment of effusions by immunocytochemistry has shown insufficient diagnostic specificity.[133] However, results can vary substantially with the method, and multiplex fluorescent immunocytochemistry is being explored.[134]

III. Equine peritoneal fluid in colic cases

A. The collection and analysis of peritoneal fluid is a common diagnostic procedure when a horse is presented because of clinical signs of colic. The results can guide clinical decisions regarding case management.

B. Because there is sufficient peritoneal fluid in the healthy horse for routine collection, it can be a diagnostic challenge to determine if the fluid results have or have not detected a pathologic state. When the results are definitely different from those expected in a healthy horse (Table 19.2, fourth and fifth columns), they are frequently typical of an exudate. However, some results are difficult to interpret.

1. Peritoneal fluid in healthy horses has protein concentrations of protein-poor transudates but often cell concentrations suggesting low-grade inflammation, i.e., greater TNCCs than expected in peritoneal fluid from healthy individuals of other species. This could be interpreted as mild inflammation, but it is not clinically significant and not evidence of disease in a horse. However, when there is an excess amount of that fluid in the peritoneal cavity, then a pathologic state is present.

2. Early in inflammation, there may be increased permeability of blood vessels to protein before there is significant migration of cells into the peritoneal cavity. At that time, an effusion's [TP_{ref}] may be increased but its TNCC may not be (for a horse), and thus the fluid has the properties of a proteinaceous exudate or protein-rich transudate. Other diagnostic information is needed to establish the pathogenesis of the effusion.

IV. Differentiation of a bacterial exudate from intestinal contents
   A. Occasionally, a collected sample contains numerous and pleomorphic bacteria that are consistent with an intestinal microbiome. The microscopist should consider that the sample might be a bacterial exudate secondary to intestinal leakage or a sample inadvertently collected, at least partially, from an intestine (called an *intestinal tap*).
   B. These samples may contain numerous bacteria and evidence of ingesta (e.g., fragments of plant fibers) and have odors and gross appearances of intestinal contents.
      1. A peritoneal sample of a bacterial exudate may have few to numerous WBCs, often degenerate, and a [TP_{ref}] > 2.0 g/dL, whereas intestinal fluid does not have many WBCs, and neutrophils, if present, tend to be nondegenerate if the sample is processed promptly. Rarely, peritoneal fluid is collected within minutes of an intestinal rupture, and then it is not possible to differentiate a peritoneal fluid sample from intestinal fluid. However, the resultant inflammatory response will be evident in samples collected a few hours later and probably sooner. If a sample is a partial collection of peritoneal fluid mixed with a partial intestinal tap, interpretation is particularly difficult, especially if the sample is not processed immediately.
      2. Even though intestinal bacteria are not pathogens within the intestine, they are recognized as foreign structures in peritoneal fluid. Accordingly, they are engulfed by neutrophils. Bacteria from an intestinal tap may undergo phagocytosis in vitro within 15–30 min after collection, even in an EDTA tube, thus complicating differentiation of a mixed peritoneal fluid–intestinal tap from a ruptured intestine (Figure 19.9C). In the authors' experience, based on purposeful contamination of fresh equine peritoneal fluid with bacilli and cocci from broth cultures, many more bacteria are found in macrophages than expected with most spontaneous septic states; most types of bacteria are found within neutrophils unless a macrophage engulfed a neutrophil that had engulfed bacteria (Figure 19.21). Exceptions include *Rhodococcus* spp. coccobacilli and nonstaining *Mycobacterium* spp. bacilli.

V. Effusions associated with neoplasms
   A. An effusion can be labeled a *neoplastic effusion* when neoplastic cells are detected in it. Most intrathoracic and intra-abdominal neoplasms that cause effusions do not exfoliate cells into the fluid; they typically cause an effusion by other processes (exudation, transudation, hemorrhage, or lymphatic damage).[135] The features of neoplastic effusions can be extremely variable, e.g., with only rare neoplastic cells among many inflammatory cells or with an extremely high concentration of neoplastic cells.
   B. Critical examination of the nucleated cells is needed to suggest or confirm the presence of neoplastic cells in an effusion. Immunocytochemistry or immunohistochemistry may be helpful to further characterize cell populations. The latter is achieved by any of several methods that convert suspended cells into cell pellets for processing as fixed tissue samples.[136] The simplest approach is gentle centrifugation of the effusion in a conical tube (as for urine sediment preparation) followed by removal of the supernatant and addition of 10 % neutral buffered formalin to the pellet in a 10:1 (formalin volume:pellet volume) ratio.

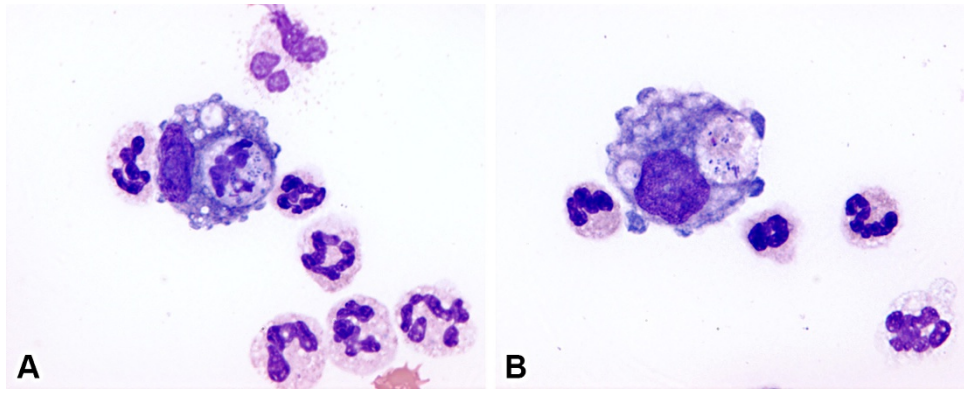


Figure 19.21. Macrophage phagocytosis of neutrophils containing bacteria. A. Neutrophils and a macrophage containing a phagocytized neutrophil that had phagocytized pleomorphic bacteria, and B. Neutrophils and a macrophage containing a partially degraded neutrophil (no nucleus) that had phagocytized pleomorphic bacteria, septic pleural exudate (pyothorax), dog. Direct smear, Wright stain, 100× objective.

1. Neoplastic lymphocytes are typically recognized when a population of cells has nuclei > 10 μm in diameter and has nuclear and cytoplasmic features of high cellular activity, although granular lymphocytes are recognized by their cytoplasmic granules and may have fewer features of cellular activity (Figure 19.8). Smaller neoplastic lymphocytes are more difficult to recognize via microscopy. Neoplastic lymphocytes may be in low to very high concentrations. They must be differentiated from cells of other hemic neoplasms including histiocytic sarcoma and acute myeloid leukemia (rare).
2. Many types of primary and metastatic carcinomas may involve abdominal or thoracic organs and exfoliate few to many cells into effusions (Figure 19.13). Such populations can be clearly malignant, but it may be impossible to differentiate them from highly reactive or neoplastic mesothelial cells by routine cytologic evaluation. Although keratinized squamous epithelial cells are often sample contaminants, they may represent neoplastic cells exfoliated from a primary or metastatic squamous cell carcinoma (Figure 19.14C). They may also be nonneoplastic cells from a ruptured esophagus or stomach (horse, Figure 19.14B). In pregnant animals, squamous cells may be from amniotic fluid that was unintentionally collected (Figure 19.22).
3. Most sarcomas do not exfoliate many cells into effusions, but atypical histiocytes of histiocytic sarcoma may be present and must be differentiated from other atypical cell populations and from inflammatory macrophages. Although hemangiomas and hemangiosarcomas of spleen, liver, lung, or heart might rupture and cause hemorrhagic effusions, neoplastic endothelial cells are rarely found in the effusions.
4. Mast cell neoplasms may be associated with few to many cells in pleural effusions, peritoneal effusions, or both. Confirmation of a mast cell neoplasm may require assessment of solid tissue containing the neoplasm, particularly with relatively few mast cells that lack atypia and therefore might be part of an inflammatory response.
5. Metastatic melanomas may damage tissues, damage lymphatic vessels, and exfoliate neoplastic cells into cavitary effusions.
6. Pleural and peritoneal mesotheliomas can produce effusions with numerous pleomorphic cells. However, it may be difficult to differentiate neoplastic mesothelial cells from carcinomatous cells or reactive mesothelial cells found in nonneoplastic effusions.

VI. Lymphocyte-rich effusions

A. *Lymphocyte-rich effusions* are those pleural and peritoneal effusions in which the dominant nucleated cell is a lymphocyte (Figures 19.7A, 19.8, 19.12B). The two most common causes of a lymphocyte-rich effusion are lymphoid neoplasia (lymphoma) and an accumulation of lymph (Table 19.5). Rarely, a low-grade inflammatory process might produce a lymphocyte-rich fluid.

B. Microscopic examination of the lymphocytes may enable a neoplastic lymphoid effusion to be differentiated from a nonneoplastic effusion. The distinguishing features include the following:

1. A largely monotypic population of lymphocytes other than small lymphocytes with their typical clumped chromatin and scant cytoplasm.
2. Nuclear diameters: If most lymphocyte nuclei are > 10 μm in diameter, then the effusion is probably neoplastic. If most lymphocyte nuclei are < 10 μm, lymphoid neoplasia may or may not be present.
3. Chromatin patterns: Dispersed or finely granular chromatin is typical of most lymphoid neoplasms.
4. Nucleoli: If many lymphocytes have nucleoli, then they probably are neoplastic; reactive (proplastic) cells may have similar nucleoli, but the lymphoid population is expected to be mixed rather than monotypic.
5. The most distinguishing feature for neoplasms of granular lymphocytes is typical cytoplasmic granulation in a monotypic population of lymphoid cells; other features vary.
6. In fluid samples, neoplastic lymphocytes can be difficult to differentiate from macrophages and, rarely, acute myeloid leukemia cells.

C. The [lymphocyte] in nonneoplastic effusions rarely exceeds $10.0 \times 10^3/\mu L$. Effusions containing neoplastic lymphocytes may have a low to very high TNCC; microscopic identification of atypical cells is more diagnostically important than the TNCC, but diagnosis of small-lymphocyte neoplasms is aided by a high lymphocyte concentration that is not expected in nonneoplastic effusions.

D. Routine analysis of a lymphocyte-rich effusion may not definitively differentiate a neoplastic from a nonneoplastic effusion. Additional diagnostic methods might include clonality studies of the collected lymphocytes, immunophenotyping, or histopathologic examination of fixed cells or associated excised tissue.

VII. Pericardial effusions

A. Pericardial effusions are less commonly evaluated than pleural and peritoneal effusions. Most are detected in radiographs and ultrasonograms (echocardiograms), most commonly in dogs. Disorders associated with pericardial effusions include neoplasms, infections, and blood or lymphatic vessel disturbances, but they frequently are idiopathic.[137–140]

B. Published descriptions of the features of pericardial fluid from healthy domestic mammals were not found. Although often assumed to be similar to pleural and peritoneal fluids in human beings and domestic mammals, recent findings for human pericardial fluid suggest it might differ by having greater total nucleated cell and protein concentrations. Pericardial fluid was collected immediately after opening the pericardial sac during elective open-heart surgery on 120 people lacking evidence of pericardial disease, decompensated heart failure, or inflammatory or metabolic disorders that might be expected to alter pericardial fluid.[141] Fluids with blood contamination were excluded. These samples were considered likely to reflect pericardial fluid in health, fluid that ethically cannot be collected. The fluids had the following characteristics (low–high intervals follow the medians):

1. The median TNCC was 1977/μL (37–8453/μL). Most of the cells were individual or clustered mesothelial cells, with a median of 1283/μL (0–5579/μL). The median [WBC] was 503/μL (22-4812/μL). Most WBCs were lymphocytes, with a median of 304/μL (8–2374/μL).
2. The median protein concentration (biuret method) was 2.8 g/dL (1.7–5.3 g/dL).
3. Although these samples were considered a proxy for samples from healthy people, the patients were not completely healthy. Also, the median age of the patients was 69 y, and 69 % were male, so age and sex bias were present. It is unknown if findings in healthy domestic mammals would be similar.

C. Pericardial fluid frequently has features of a hemorrhagic effusion. In such samples, macrophages containing RBCs, hemosiderin, and hematoidin (bilirubin) crystals are common. In a retrospective review of 259 canine pericardial fluids, 90 % were hemorrhagic (idiopathic), 5 % neoplastic, 3 % infectious, and 2 % chylous or low-cellularity effusions.[142] When effusions were classified as neoplastic or infectious, they were not considered hemorrhagic, even if bloody. The diagnostic value of fluid analysis was better for fluids with a Hct < 10 %.

D. Infections are usually bacterial and rarely fungal (e.g., candidiasis) or protozoal (e.g., leishmaniasis, toxoplasmosis). They may arise hematogenously or by extension from adjacent tissue or a migrating foreign body. FIP may also cause pericardial effusions.

E. Neoplastic cells are uncommonly detected, even when a neoplasm is associated with the effusion (e.g., chemodectoma, thyroid carcinoma, hemangiosarcoma). However, atypical cells may be present and supportive or diagnostic of neoplasms including lymphomas, histiocytic sarcomas, mast cell neoplasms, and carcinomas. Cells of a mesothelioma may be impossible to differentiate from reactive mesothelial cells.
1. Mesothelial cells in pericardial effusions can have marked reactive (proplastic) changes, including intense cytoplasmic basophilia, nuclei with distinct nucleoli, and variable nuclear to cytoplasmic ratios. These cells should not be considered neoplastic cells even though their microscopic features are similar to malignant cells. Rarely, mesothelial cells contain uniform iron-containing granules that are golden-brown with routine stains and blue with Prussian blue staining. They might also contain bilirubin.
2. Although some data have suggested that the pH of pericardial fluid may help differentiate pericardial effusions due to neoplasms from other causes, individual cases could not be reliably differentiated by the pH values.[143,144] As with measurement of blood pH (see Chapter 10), sample handling and processing are critical to obtain accurate pH values.

F. A chylous pericardial effusion may be secondary to chylothorax or occur alone.[145] Eosinophilic exudates are rare and suggest the same types of disorders as for peritoneal or pleural fluids.

VIII. Amniotic fluid

A. Occasionally, amniotic fluid is inadvertently collected during paracentesis.

B. Unless there is a pathologic state involving a fetus or the amniotic sac, the amniotic fluid is clear and watery, and microscopic examination reveals numerous nucleated or anucleate superficial squamous epithelial cells (Figure 19.22).

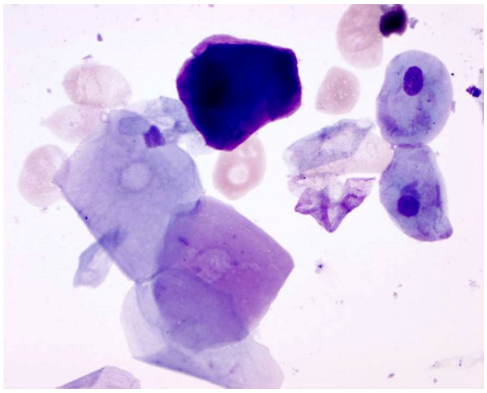


Figure 19.22. Amniotic fluid. Nucleate and anucleate squamous epithelial cells in amniotic fluid collected inadvertently during attempted abdominocentesis, alpaca. Cytocentrifuge preparation, Wright stain, 100× objective.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 20

SYNOVIAL FLUID

Michael M. Fry, Michael A. Scott, and Steven L. Stockham

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Table 20.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
ANA	Antinuclear antibody
EDTA	Ethylenediaminetetraacetic acid
Ig	Immunoglobulin, as in IgG (immunoglobulin G), IgM, and IgA
IMPA	Immune-mediated polyarthritis
LE	Lupus erythematosus
OA	Osteoarthritis
RBC	Red blood cell (erythrocyte)
RF	Rheumatoid factor
TNCC	Total nucleated cell concentration
TP	Total protein
TP_{ref}	Total protein determined by refractometry
WBC	White blood cell (leukocyte)

GENERAL CONCEPTS AND DEFINITIONS

I. Synovium, also referred to as synovial membrane, lines diarthrodial (synovial) joints, bursae, and tendon sheaths. It encloses *synovial fluid* or *synovia* (resembling egg white – from L., Gr. *syn* with + *ōon* egg), which is sometimes referred to more specifically as joint fluid, bursal fluid, or tendon sheath fluid (Figure 20.1).

II. Synovial fluid in health[1–4]

A. Synovial fluid is a filtrate of plasma through subintimal capillaries, modified by additions from the synovium. Synovial fluid constituents originating from plasma include proteins, glucose, urea, and electrolytes. Production and absorption of synovial fluid occurs continually, influenced by Starling forces and intra-articular pressure. Removal of fluid from the joint cavity occurs via the synovial lymphatic system.

B. Synovial fluid performs important biomechanical and metabolic functions. Cushioning and lubricant functions of synovial fluid are highly dependent on hyaluronic acid and lubricin (proteoglycan 4), respectively. Fluid moves between the joint space and articular cartilage in response to weight bearing, which promotes nutrient delivery to, and waste removal from, cartilage.

C. The volume of synovial fluid in health varies by species and anatomic location. Joints of healthy dogs and cats commonly yield less than 0.5 mL,[5] sometimes a small drop or none, whereas commonly sampled joints of cattle and horses typically yield at least 2 mL and may yield as much as 20 mL (equine tarsus).[6]

D. Synovial fluid has lower total nucleated cell and neutrophil concentrations than blood. Published data reflecting expected protein and TNCCs of synovial fluid in healthy dogs, cats, horses, and camelids are summarized in Tables 20.2–20.4. Results are limited by varied analytical methods, varied cell classifications, assessment of different joints, and the number of joints assessed. There are very few published data for features of synovial fluid from cattle, and most are from postmortem samples. In three publications, the TNCCs were < 700/μL; TP data were not reported.[7–9]

III. Definitions

A. See the General Concepts and Definitions section, Chapter 19 (Cavitary Effusions), for definitions of *effusion*, *exudate*, and *hemorrhage*.

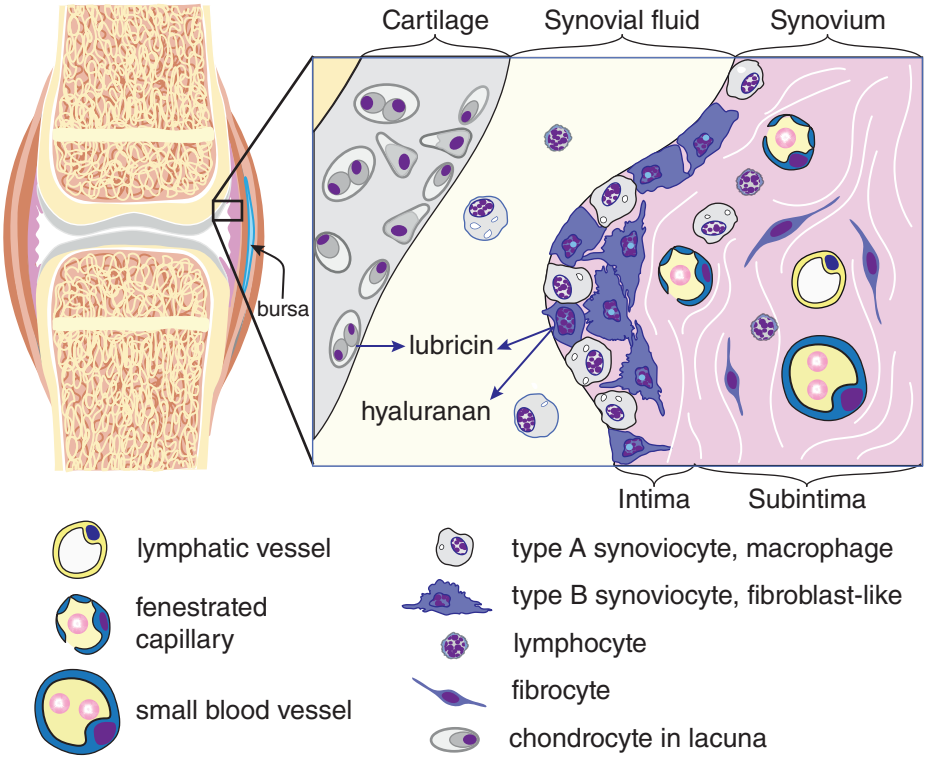


Figure 20.1. Major components of a generic synovial joint and its synovium and fluid.

- Synovial fluid is enclosed by articular cartilage and synovium, the latter being surrounded by the fibrous components of the joint capsule. Synovium forms evaginations called villi and consists of a superficial intima and deeper subintima.
- The subintima is vascular connective tissue containing small blood vessels, lymphatic vessels, nerve fibers, adipocytes, cells of the immune system (macrophages, lymphocytes, fibrocytes, plasma cells, dendritic cells, mast cells), and extracellular matrix.
- The intima lacks a basement membrane and is composed of Type A and Type B synoviocytes. *Type A synoviocytes* are specialized macrophages thought to originate from monocytes migrating from subintimal, postcapillary venules; they are believed to function in uptake and metabolism of molecules in synovial fluid. *Type B synoviocytes*, which predominate, are fibroblast-like cells rich in endoplasmic reticulum.
- Synovial fluid is continually produced as a synovium-modified filtrate of plasma from subintimal fenestrated capillaries. Type B synoviocytes synthesize and secrete hyaluronan and lubricin (proteoglycan 4); they may also regulate movement of inflammatory cells into the fluid, where low concentrations of mostly macrophages and lymphocytes are present in health. Synovial fluid carries nutrients to cartilage and wastes from cartilage and chonodrocytes back to the synovium for removal by macrophages and lymphatic vessels. Hyaluronan and lubricin, the latter also produced by chondrocytes, together reduce friction, lubricate the joint, and contribute to shock absorption.

B. *Arthropathy* is a term for all forms of joint disease.

C. *Arthritis* refers to inflammation involving a joint and, according to some sources, implies a lesion involving articular cartilage.[1]

D. *Osteoarthritis* (OA), also known as *degenerative joint disease* or *osteoarthrosis*, is an arthropathy characterized by progressive deterioration or loss of articular cartilage. Recognized initiators

Table 20.2. Reported features of synovial fluid samples from healthy dogs

	Atilola (1986)	Atilola (1984)[a]	Sawyer[b]	Fernandez[c]
TNCC (/μL)[d]	200–1700[e] 300–2100[e]	700–4400	0–2900	0–1500
Neutrophils (%)	—	4	0–12	0–11 (0–19)[c]
Other cells (%)[f]	—	96	88–100	89–100 (81–100)[c]
[TP] (g/dL)	—	2.6 (± 1.1)	—	1.8–4.8
Number of samples	19	20	55/64[b]	19
Number of dogs	19	12	46	15

[a] TNCC results for stifle-joint samples were determined by electronic cell counting using diluted samples. Mean percentages of differential cell counts were reported. The method of [TP] measurement was not reported, nor was it reported whether the (± 1.1) was for one or two standard deviations, but other data were reported as ± one standard deviation. Samples with blood contamination were excluded.[10]

[b] TNCC results are for samples from carpal, elbow, hip, hock, shoulder, and stifle joints, and were determined by hemocytometer counting. The greatest [RBC] was 0.3×10^6/μL. There were 55 samples for TNCC and 64 for cell percentages.[5]

[c] TNCC results are for samples collected from carpal, shoulder, and stifle joints, and were determined by hemocytometer counting. Differential count percentages are for nine direct smears; minor differences were reported for stop-flow and cytocentrifuge preparations of hyaluronidase-treated samples, with up to 19 % neutrophils in the latter (parenthetic data). [TP] was determined by refractometry on EDTA samples; the 4.8 g/dL result was from a very viscous stifle joint fluid. RBC data were not reported.[11]

[d] Reported cell concentrations are rounded to the nearest 100/μL.

[e] TNCC results are for stifle-joint samples (one outlier excluded). The first row of data was determined by electronic cell counting, whereas hemocytometer counting was used for the second row of data;[12] diluted samples were used for both methods.

[f] Nucleated cells reported as monocytes, macrophages (clasmatocytes), synoviocytes (synovial-lining cells), lymphocytes, and miscellaneous cells were combined into "Other cells" because of inconsistent classification of cells other than neutrophils.

of OA include abnormal joint mechanics (conformation, congruence, stability, bone stiffness), osteochondrosis, direct trauma to articular cartilage, and synovitis. Inflammation is not likely to be an essential inciting cause of OA, but inflammatory cytokines and other mediators may be present in increased concentration in joints affected with OA.[20,21]

E. *Hemarthrosis* means bleeding into a joint.

F. *Synovitis* is inflammation of the synovium.

IV. Clinical indications for obtaining a synovial fluid sample

A. Presence of a palpable joint effusion: The goal is to reach a diagnosis, better characterize a disease process, or monitor a disorder. The sample is often collected to assess for evidence of immune-mediated disease or infection.

B. Assessment for polyarthritis: When one joint is clearly affected, other joints may be sampled whether or not they have discernible effusions.

C. Unexplained lameness (especially shifting leg lameness) or joint pain in the absence of any clinical findings to explain it: The goal is to determine if there is evidence of joint disease, but the absence of definitive synovial fluid abnormalities does not exclude it.

D. Fever of unknown origin (FUO): Sampling is done to look for an occult inflammatory arthropathy.

Table 20.3. Reported features of synovial fluids of healthy horses

	Van Pelt[a]	Persson[b]	Dykgraaf[c]	Malark[d]	Tulamo[e]
TNCC (/μL)[f]	≤ 1400	≤ 500	≤ 800	≤ 3500	≤ 300
Neutrophils (%)	0–52	—	0–28	0–20	—
Other cells (%)	48–100	—	72–100	80–100	—
RBC (/μL)	—	< 500,000	< 100,000	< 100,000	—
TP (g/dL)	—	0.4–2.2	1.2–2.3	0.4–1.5	< 2.5
Number of samples	87/63	113	16	52	6
Number of horses	27	—	8	13	6

[a] Results are for samples from five different joints; data from the various joints are combined as there were not major differences among sites. TNCCs were determined by hemocytometer for 87 samples; differential counts were determined for 63 samples. RBC and TP data were not reported.[13]

[b] Results are for samples from various joints of an unspecified number of horses "of standard breed" and "Swedish half-breed" from stud and trotting tracks of Sweden. TP concentrations were determined for 233 samples by biuret assay. TNCCs were determined by hemocytometer. Differential cell counts were not reported.[14]

[c] Results are for samples from digital flexor tendon sheaths; TNCCs were determined by electronic counter, and TP concentrations were determined by refractometer.[15]

[d] Results are for samples from digital flexor tendon sheaths; TNCCs were determined by electronic counter, and TP concentrations were determined by Coomassie brilliant blue assay.[16]

[e] Results are for samples from tarsocrural joints. TNCCs were determined by hemocytometer, and TP concentrations were determined by refractometer.[17]

[f] Cell concentrations are rounded to the nearest 100/μL.

Table 20.4. Reported features of synovial fluid from healthy cats, llamas, and alpacas

	Cats[a]	Llamas[b]	Alpacas[b]
TNCC (/μL)[c]	≤ 1100	≤ 1200	≤ 1400
Neutrophils (%)	0–39	0–4	0–12
Other cells (%)[d]	61–100	96–100	88–100
RBC (/μL)	≤ 4500	—	—
TP (g/dL)	—	2.0–3.8	2.1–2.5
Number of samples	126	?	?
Number of animals	?	15	15

[a] TNCC results for stifle and shoulder joint samples were determined by hemocytometer counts; the number of cats for the samples was not specified.[18]

[b] TNCC results are for samples from radiocarpal and tarsocrural joints; they were determined with an electronic cell counter. TP concentrations were determined by refractometry; a total of 49 samples from llamas and alpacas were analyzed.[19]

[c] Reported cell concentrations are rounded to the nearest 100/μL.

[d] Nucleated cells reported as monocytes, macrophages (clasmatocytes), synoviocytes (synovial-lining cells), lymphocytes, and miscellaneous cells were combined into "Other cells" because of inconsistent classification of cells other than neutrophils.

CLASSIFICATION OF SYNOVIAL EFFUSIONS

I. There is no generally accepted system for classifying synovial effusions using objective criteria. Most abnormal synovial fluid samples have been classified into one of two major categories variously termed suppurative or nonsuppurative, purulent or nonpurulent, exudative or nonexudative, and inflammatory or noninflammatory arthropathies.
   A. The terms *suppurative*, *purulent*, *exudative*, or *inflammatory* have been used when the neutrophil concentration is increased.[22–26]
   B. When macrophages predominate and neutrophils are not considered a significant inflammatory component, the terms *nonsuppurative*, *nonpurulent*, *nonexudative*, and *noninflammatory* have been applied.
   C. These categories are not used here because:
      1. Most definitions of the terms *suppurative* and *purulent* relate to the formation of pus, which the vast majority of synovial fluid samples do not resemble.
      2. Categorizing OA as a degenerative or noninflammatory condition as opposed to an inflammatory condition is not consistent with the term (*-itis*) or the low-grade inflammation occurring in OA.[27,28]
      3. The term *exudative* does not differentiate proteinaceous exudates from cellular exudates; a fluid with low nucleated cell concentration but increased protein concentration could be exudative, but the causes of such fluids are not the same as those of effusions with marked neutrophilic components.

II. Classification of synovial fluid abnormalities in this book
   A. When possible, classification is based largely on a fluid's predominant cell types, including inflammatory cells, neoplastic cells, and RBCs (Table 20.5). Recognizing an increased concentration of any nonneoplastic nucleated cell type requires consideration of the TNCC, the percentage of that cell type, and potential contributions of blood contamination. An increased concentration of a cell type is present when both TNCC and the cell's percentage are increased, and sometimes when either one is increased alone (e.g., 80 % neutrophils with TNCC near but below the upper reference limit, or 15 % neutrophils with a moderately increased TNCC). See the last major section in the chapter for more detailed information about specific types of arthropathies.
      1. *Neutrophilic arthropathies* are those in which the predominant cellular abnormality is an increased concentration of neutrophils not attributable to hemorrhage. Neutrophils are usually the majority of nucleated cells, but not always, such as in mild or resolving inflammatory states with relatively low TNCCs.
      2. *Macrophagic arthropathies* are those in which the predominant cellular abnormality is an increased concentration of macrophages or the presence of macrophages that clearly show their participation in an inflammatory process (e.g., intracellular yeast or foreign material). A macrophagic arthropathy may also be present when macrophages predominate without an increased TNCC or clear cytologic abnormalities supporting inflammation, but this conclusion relies on clinical evidence of an arthropathy.
      3. *Lymphocytic or lymphoplasmacytic arthropathies* are those in which an increased concentration of lymphocytes, or lymphocytes and plasma cells, is the predominant cellular abnormality. Lymphocytes may or may not constitute the majority of nucleated cells.
      4. *Eosinophilic arthropathies* are those in which the predominant cellular abnormality is an increased concentration of eosinophils. Eosinophils may or may not constitute the majority of nucleated cells.

Table 20.5. Disorders and conditions that cause synovial fluid abnormalities

Neutrophilic arthropathies[a]
Joint infections*
Bacterial* (many species reported, including *Borrelia burgdorferi*)
Other (rare): fungal (*Blastomyces, Histoplasma*), protozoal (*Leishmania*, *Trypanosoma*)
Arthropathies known or suspected to be immune mediated (usually polyarticular)
Immune-mediated polyarthritis*
Systemic lupus erythematosus
Rheumatoid arthritis
Other: includes several specific syndromes, breed-associated polyarthritides (akitas, greyhounds), vaccine or drug reaction (e.g., sulfonamides)
Trauma
Joint injections / foreign material
Pancreatitis, panniculitis, and polyarthritis syndrome
Shar pei autoinflammatory disease (swollen-hock syndrome, familial amyloidosis, shar pei fever)
Macrophagic arthropathies
Osteoarthritis* (many causes)
Foreign material (silicone) (rare)
Repeated arthrocentesis
Lymphocytic arthropathies (rare, see the text)
Eosinophilic arthropathies (rare)
Idiopathic (suspected immune mediated)
Injection reaction
Hemorrhagic arthropathies
Mixed inflammatory cell arthropathies
Trauma*
Coagulopathies
Neoplastic arthropathies (rare)
Primary neoplasm affecting a joint
Metastasis to a joint

* A relatively common disorder, condition, or mechanism

[a] Fluids from animals with conditions in these categories may be classified as mixed inflammation and subclassified further according to the particular cell types involved, because cell proportions and concentrations vary with the degree of inflammatory stimulus, the duration of the inflammation, treatment effects, and individual biological variation.

5. *Mixed-inflammatory-cell arthropathies* are those in which there are prominent abnormalities of more than one cell type.
   a. They may be subclassified according to the particular cell types involved, e.g., mixed inflammation with neutrophilic and lymphocytic components.
   b. Cell proportions and concentrations vary with the nature, magnitude, and duration of inflammatory stimulus, treatment effects, and individual biological variation.
6. *Hemorrhagic arthropathies* are those in which bleeding into the joint is the predominant abnormality.

7. *Neoplastic arthropathies* can be considered to include any arthropathy caused by a neoplasm, but in the context of classification based solely on synovial fluid analysis, neoplastic cells must be present to be able to identify and classify the disorder as a neoplastic arthropathy.

B. Reported features of synovial fluid vary with species, may vary with the joint, and are not well established (Tables 20.2–20.4), so recognizing mild deviations from health is problematic. The following may be the only conclusions that can be reached by synovial fluid analysis in some cases:
   1. "There is no definitive evidence of disease, but mild neutrophilic inflammation might be present." Causes of neutrophilic and mixed neutrophilic/macrophagic inflammation should be considered, but it is not clear that disease is present.
   2. "There is no definitive evidence of disease, but mild macrophagic inflammation might be present." Causes of macrophagic inflammation should be considered, but it is not clear that disease is present.

C. When no definitive evidence of disease is apparent upon analysis of synovial fluid, the joint may or may not be healthy.

PATHOGENESES OF SYNOVIAL EFFUSIONS

Synovial effusion occurs when one or more pathologic processes cause increased entry of fluid into, or decreased removal of fluid from, the joint space. Fluid, protein, and cells may enter a synovial joint because of inflammation or hemorrhage, and impaired lymphatic drainage may reduce drainage. Although specific mechanisms contributing to synovial effusions and their composition have not been determined for many disorders, analysis of an effusion generally allows classification into a general pattern and major pathologic process; then, other information (history, physical examination, results of imaging, or other laboratory testing) is used to determine a specific disorder (Table 20.5). Common features of synovial fluid for different types of arthropathies are shown in Table 20.6.

I. Inflammation

A. Increased vascular permeability to proteins and exudation of fluid from the vasculature are hallmarks of acute inflammation. Inflammation in a joint can cause synovial blood vessels to become more permeable, allowing fluid, protein, and cells to exude into the joint space. See the Pathogenesis of Cavitary Effusions section, Chapter 19, for more detailed discussion of mechanisms of exudation.

B. Synovial inflammation and associated effusions can be caused by infection, immune-mediated disease, tissue degeneration, trauma, neoplasms, crystal deposition, and intra-articular injections of foreign material. Joints can become infected hematogenously,[29–31] traumatically (including surgically),[32] or by extension from an adjoining site.

C. In immune-mediated joint disease, immune complex deposition in the synovium may elicit an inflammatory reaction (type III hypersensitivity reaction) through complement activation and binding to Fc receptors on macrophages. Complement opsonization of immune complexes may promote their phagocytosis and formation of so-called LE cells (see III.D.2 in Routine Analysis of Synovial Fluid).[33] The immune complexes may form because of autoimmune disease or in response to infection or other antigenic stimulation (e.g., drug reactions) elsewhere in the body, the joints themselves being sterile.[34,35] In rheumatoid arthritis, certain autoantibodies may promote damage by binding directly to tissue (e.g., to osteoclasts),[36]

Table 20.6. Common features of synovial fluid for different types of arthropathies[a]

Arthropathy classification	Predominant cell	TNCC ($\times 10^3/\mu L$)	Comments
Neutrophilic	Neutrophil	5 to > 100	Likely turbid; evidence of infection (organisms) or immune-mediated arthritis (inclusions) in a minority
Macrophagic	Macrophage	< 5	Increased [macrophage] and/or evidence of macrophage role (e.g., intracellular organism or foreign material)
Lymphocytic (rare)	Lymphocyte	?	Increased [lymphocyte] ± plasma cells
Eosinophilic (rare)	Variable	3 to > 10	Increased [eosinophil] but neutrophils and macrophages may predominate
Mixed inflammatory	Variable	Variable	Prominent increase in concentrations of more than one inflammatory cell type
Hemorrhagic	RBC	Variable	Pink to red grossly ± hemosiderin or RBCs in macrophages; hemorrhage is the predominant abnormality
Neoplastic (rare)	Variable	Variable	Neoplastic cells ± other changes

[a] Descriptions of common findings are not comprehensive, and some cases may have values outside of the thresholds shown.

and chronic synovial membrane proliferation over articular cartilage (pannus) promotes articular cartilage erosions, adjacent bone resorption, and joint deformity.

D. Research of *Borrelia*-related arthropathy in people and mouse models suggests that synovitis may continue after antibiotic clearance of live organisms because of persistent spirochetal antigens and various types of infection-induced autoimmunity other than Type III hypersensitivity.[37]

E. Fluid exudation is often accompanied by migration of WBCs into the effusion in response to chemotactic signals. The number and type of cells recruited into the fluid vary depending on the specific inflammatory stimuli. Synovial fluid findings do not always correlate well with synovial tissue lesions. For example, inflammation or neoplasia may be present in the synovium without being evident in the synovial fluid,[38,39] and different inflammatory cell types may predominate in the synovium and the synovial fluid.[40]

II. Impaired lymphatic drainage: Absorption of fluid by synovial lymphatic vessels is part of joint homeostasis, so lymphatic damage or obstruction can promote synovial effusions. Decreased numbers of lymphatic vessels may play a role in development of synovial effusion in some individuals with OA.[41–43]

III. Hemorrhage: Bleeding into the joint space can occur because of trauma, acquired or congenital bleeding tendencies, or neoplasms. Minor hemorrhage often accompanies exudation because of inflammation-induced vascular damage. See the Pathogenesis of Cavitary Effusions section, Chapter 19, for more detailed discussion of hemorrhagic effusions.

ROUTINE ANALYSIS OF SYNOVIAL FLUID

Results of analysis of synovial fluid may indicate a specific cause for the effusion. More often, however, the results shorten the list of possibilities being considered to explain the effusion. Usually, the most important initial clinical determination based on routine testing of synovial fluid is whether there is evidence of neutrophilic inflammation. Characteristic findings for different types of joint disease are discussed later (Comments about Specific Types of Arthropathies).

I. Sample collection and processing
   A. Synovial fluid samples should be collected using aseptic technique to avoid microbial contamination of the joint. Description of recommended arthrocentesis techniques is beyond the scope of this chapter.
   B. Sample volume may limit analysis and require prioritization of testing, especially for smaller joints and joints with no or minimal palpable effusion. In general, the top test priority is microscopic examination of the fluid.
   C. If the volume of sample collected is sufficient, synovial fluid should be placed into different tubes, depending on the analyses to be performed.
      1. A tube containing EDTA anticoagulant is generally preferred for routine synovial fluid analysis, because it reduces the possibility of fibrin clot formation secondary to exudation or bleeding. Clots compromise analysis. A plain clot tube may be used when clotting is unlikely. Most laboratories prefer to receive at least 1 mL of fluid when possible; a tube of appropriate size should be used, as excess EDTA-fluid constituents can falsely increase protein concentration determined by refractometry.[44–46]
      2. Sample aliquots to be submitted for culture should be placed (using aseptic technique) in a sterile tube without anticoagulant, or directly into a blood culture tube or bottle. Samples to be submitted for culture should not be put into EDTA, because it can inhibit microbial growth.
   D. Direct smears of fluid should be made immediately, particularly if sample analysis will be at all delayed. With delays of more than a few hours, the rest of the fluid should be refrigerated or kept cool (e.g., for mailing) to limit cell deterioration that can affect TNCC and microscopic evaluation of preparations made later at a laboratory. Direct smears are made with a wedge or gentle squash technique, both of which are easy and inexpensive. However, direct smears generally contain fewer well-spread cells for microscopic evaluation than do concentrated preparations. Concentrated preparations are typically made by cytocentrifugation at reference laboratories, but line preparations (flow-back smears) or sediment smears can also be used. The best preparations for cellular detail are commonly made from hyaluronidase-treated fluid.
      1. Treating synovial fluid with hyaluronidase (available in lyophilized form) enzymatically degrades hyaluronic acid and changes the consistency of the sample from viscous to watery. Cells in hyaluronidase-treated fluid disperse and flatten better than those in smears made from untreated fluid, thus allowing better assessment and more reliable differentiation of cell types.[47]
      2. Hyaluronidase may be added by dipping a wooden applicator stick into a vial of lyophilized hyaluronidase to coat the tip of the stick with enzyme, then swirling the stick in a tube containing the synovial fluid sample. This does not significantly alter the sample volume or dilute the cells. Another method is to make a hyaluronidase solution in buffered saline and mix that with the synovial fluid, a process that can be done quantitatively to allow for automated measurement and back-calculation of cell concentrations.[48]

E. Romanowsky-type staining of direct or concentrated cytologic preparations is used routinely as for other cytologic and hematologic samples.

II. Gross evaluation of synovial fluid

A. In health, synovial fluid is clear, colorless to slightly yellow, viscous, and free of discernible solids. It is *thixotropic*, meaning it may become more fluid when shaken or agitated, and more gel-like when allowed to rest. Shaking or hyaluronidase treatment may be needed to liquefy a sample enough to analyze it.

B. A grossly abnormal appearance predicts that other abnormalities will be detected during complete fluid analysis, but a normal appearance does not exclude the possibility of abnormal findings. Causes of abnormal transparency, color, or viscosity are provided in Table 20.7.

C. Transparency is commonly assessed by evaluating the appearance of printed material through the fluid in a clear tube. Decreased clarity of the printed material indicates a degree of turbidity that may be described (hazy, opaque) or graded (e.g., 1+ to 4+, with 4+ reflecting unreadable print).

1. Increased turbidity typically signifies an increase in cell concentration(s).
2. Other particles such as organisms or crystals can also contribute to turbidity, but solutes such as protein do not.

D. Color is assessed by observing the fluid in a clear tube.

1. Whitish discoloration occurs if fluid contains a sufficient concentration of WBCs or less commonly neoplastic cells, organisms, or potentially crystals.
2. Pink or red discoloration occurs if fluid contains a sufficient concentration of RBCs (whether due to iatrogenic or pathologic hemorrhage), or free hemoglobin from RBCs lysed in vivo or in vitro. A red-brown or brown color may occur with older hemorrhage and hemoglobin derivatives including methemoglobin, bilirubin, and hemosiderin.
3. Yellow or orange discoloration, called *xanthochromia* (from Greek *xanthos* yellow and *chroma* color) reflects increased bilirubin concentration, and occurs as a result of hemoglobin degradation due to prior hemorrhage in the joint. Similar discoloration could occur due to hyperbilirubinemia.

Table 20.7. Causes of abnormal transparency, color, and viscosity

Increased turbidity
Increased TNCC (mostly WBCs)*
Increased [RBC]*
Other suspended particles (foreign material, crystals, organisms, cellular debris)
Abnormal colors
Whitish:* increased TNCC; many organisms or crystals (rare)
Pink to red:* RBCs or free hemoglobin
Yellow to orange: presence of bilirubin
Brown or red-brown: combinations of RBCs, free hemoglobin, methemoglobin, bilirubin, hemosiderin
Decreased viscosity
Decreased production of hyaluronan or lubricin by Type B synoviocytes or chondrocytes*
Degradation of hyaluronan or lubricin by enzymes from inflammatory cells or bacteria*
Dilution of synovial fluid by exudation, hemorrhage, or lavage

* A relatively common disorder, condition, or mechanism

E. Viscosity of synovial fluid is assessed by observing its consistency. Synovial fluid of normal viscosity is stringy instead of watery.
   1. Although viscometers or rheometers have been used to quantify synovial fluid viscosity and flow, results vary with the method, particularly because synovial fluid viscosity increases as shear rate decreases, and the authors are not aware of such instruments being used in veterinary medicine. Instead, some laboratories assess viscosity by measuring the length of the string that forms when a small amount of synovial fluid is placed between the gloved thumb and forefinger and the digits are then pulled apart. Other laboratories measure the length of the string that forms when a drop of fluid is expelled from a pipette or from a needle attached to a syringe. With any of these approaches, normal synovial fluid should form a string at least one inch long before it breaks.[24]
   2. Joint disease can cause decreased viscosity of synovial fluid by degrading, diluting, or altering production of hyaluronan or other molecules.[49–53] Neutrophilic inflammation can cause decreased viscosity due to degradation of hyaluronan or glycoproteins by enzymes from inflammatory cells; bacterial enzymes may have similar effects.
   3. Increased viscosity is not commonly considered, but it was reported for synovial fluid from multiple effusive joints of a 2-yr-old lame dog with hypothyroidism.[54] Synovial fluid smears did not suggest an increased TNCC, and "mononuclear" cells predominated. The arthropathy had improved after 3 wk of levothyroxine administration and was resolved at 6 mo. Increased synovial fluid viscosity has similarly been reported in a subset of hypothyroid people with OA.

F. Fibrin clots are detected visually as "lumps" of material suspended in the fluid. Whether formed in vitro or in vivo, fibrin clots invalidate (falsely decrease) the protein and cell concentrations of the fluid because some cells and protein are incorporated into the clots. Clots can be evaluated cytologically via squash preparations as part of the microscopic analysis.

III. Microscopic examination: Microscopic examination is used to assess cell appearances and proportions, crudely estimate cell concentrations, and detect alterations to the extracellular matrix or presence of clinically relevant extracellular structures such as organisms, crystals, or foreign material. Microscopic examination is often considered the most important part of synovial fluid analysis and should be given high priority when low sample volume limits testing.

A. Major aspects of the examination
   1. Subjective microscopic assessment of glycoproteinaceous content requires a direct smear of sample without hyaluronidase treatment.
      a. This may be the only means of assessing viscosity (indirectly) if the sample is limited to a slide preparation and no fluid is available.
      b. Stained with a Romanowsky-type stain, smears of synovial fluid from healthy animals have dense and usually pink stippled to homogeneous staining in the extracellular spaces, often with protein crescents in the thickest regions (Figure 20.2). This staining pattern is due largely to the anionic glycosaminoglycan, hyaluronan, which also imparts much of the fluid's viscous consistency. The viscosity of the fluid often causes cells to line up in rows, and cells are typically sphered ("balled up") and unrecognizable (Figure 20.2) except in the thinnest areas (edges) of the smear.
      c. Reduced glycoproteinaceous content with reduced cell rowing and cell sphering support disease. Causes include enzymatic breakdown of hyaluronan by cells or organisms, and dilution secondary to an effusive condition or joint flushing. There is in vitro evidence that WBC myeloperoxidase might decrease synovial fluid viscosity through oxidative injury to hyaluronan.[55]

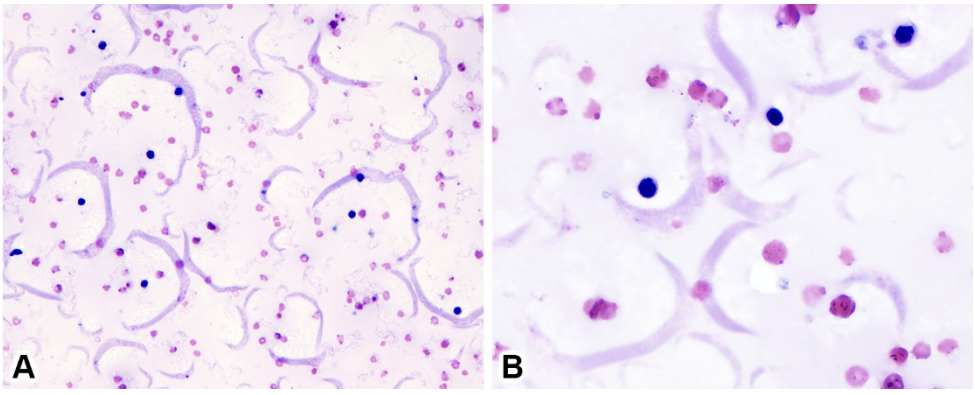


Figure 20.2. Glycoprotein crescents in a thick region of a direct smear of synovial fluid from a dog with IMPA. Note that nucleated cells, which were mostly neutrophils in this sample, are difficult or impossible to identify because they are "balled up" (diameter of RBCs) in this region. 20× objective (A) and 50× objective (B), Wright stain.

2. Assessment of cell concentrations
   a. Slide evaluation may be the only means of assessing cell concentrations if the sample is limited to a slide preparation and no fluid is available (see Section IV, Total Nucleated Cell Concentration).
   b. Subjective assessment of cellularity as increased or within limits of health is common but prone to error. Use of a time-consuming standardized method developed to estimate TNCC from direct smears of hyaluronidase-untreated canine synovial fluid correctly placed 78 % of 58 samples into the following three TNCC categories: (i) within limits of health (defined as < 3000/μL), (ii) mildly to moderately increased (defined as 3000–20,000/μL), or (iii) markedly increased (defined as > 20,000/μL).[56] TNCC tended to be overestimated when low and underestimated when high. Samples with significant hemorrhage were not evaluated. The degree to which this method is reproducible is unknown.
   c. Any major discrepancies between microscopic assessment of TNCC and cell concentrations determined by automated methods should prompt re-evaluation of the automated values, though automated values are generally expected to be more accurate than microscopic estimates.
3. Nucleated cell differential count
   a. Microscopic evaluation is needed to determine proportions of different nucleated cell types. When the cells are well spread, this can be done via objective enumeration or by subjective estimate. In thick smears, differentials may be unreliable, and lymphocytes may be overestimated because macrophages and even neutrophils may resemble them (because they are balled up).
   b. The differential count is interpreted in the context of the TNCC so that the concentrations of each cell type can be considered; 15 % neutrophils with a TNCC of 400/μL may occur in health, whereas 15 % neutrophils with a TNCC of 4000/μL or 40,000/μL reflects neutrophilic inflammation. Contributions of WBCs in any contaminating blood are also subjectively factored into the interpretation of the TNCC and individual cell type percentages and concentrations.

4. Evaluation of cell appearances: Cells are evaluated for abnormal features indicative of disease. For example, the presence of intracytoplasmic inclusions may indicate infection or immune-mediated disease, and atypical cell populations may indicate a neoplasm.
5. If present, extracellular structures such as microorganisms, crystals, or foreign material are identified.

B. Cells commonly identified in synovial fluid
1. Macrophages are the predominant cell type in health. Some people classify them as "large mononuclear cells," which would also encompass any similar-appearing type B synoviocytes. They have been called *clasmatocytes*.
   a. Macrophages are the predominant nucleated cell type in fluid from joints with OA, and they are often present in increased concentrations in effusions in which other inflammatory cells predominate.
   b. Macrophages may contain phagocytized cells, foreign material, or microorganisms, but bacteria are usually present in neutrophils.
2. Lymphocytes are often present in health, usually as a minority of nucleated cells.
   a. Lymphocytes may be present in increased concentrations in effusions in which other inflammatory cells predominate, and in rare cases may themselves predominate. They may appear reactive.
   b. Lymphocytes may be overestimated in thick smear preparations because other cells ball up and appear lymphoid with round nuclei and scant cytoplasm.
3. Neutrophils may be present in low concentration in health, as reflected by low to moderate neutrophil percentages combined with low TNCCs (Tables 20.2–20.4).
   a. Neutrophil concentration is mildly to markedly increased in many effusions, most commonly those caused by immune-mediated joint disease or infection.
   b. Neutrophil percentages and concentrations may be artifactually increased by hemorrhage during sample collection.
   c. If a sample contains blood, whether due to sampling-related contamination or hemorrhage prior to sampling, the presence of increased neutrophils should be interpreted with consideration of the amount of blood and the patient's blood leukogram; however, it may be impossible to determine with certainty whether neutrophilic inflammation is present in the joint.
4. RBCs are present in many samples as a result of iatrogenic (sampling-related) hemorrhage, but pathologic hemorrhage may also be the cause (see Hemorrhagic Arthropathies later in the chapter, as well as Chapter 19 for findings and considerations associated with pathologic hemorrhage).
5. Platelets indicate iatrogenic blood contamination, active or very recent bleeding into the joint, or both.

C. Other cells that may be found in synovial fluid include other WBCs, synoviocytes, neoplastic cells, multinucleate giant or osteoclast-like cells, osteoclasts, osteoblasts, and chondrocytes. WBCs and neoplastic cells may be present as part of blood contamination or because of local exudation or neoplastic proliferation. Bone and cartilage cells may be present because of traumatic collection or a pathologic state. The significance of multinucleate giant cells is unclear; they have been shown to be present in increased numbers in the synovial membrane of human patients with a variety of arthritides, particularly OA and rheumatoid arthritis.[57]

D. Other findings
1. Organisms (see Chapter 19 for more information on the microscopic appearance of organisms)

a. Some organisms may be found in synovial fluid because of blood contamination rather than local infection (e.g., microfilariae). Others, like *Ehrlichia ewingii* in acute infections, may enter the joint via infected inflammatory cells that migrate to the joint because of disease caused by the organism but not because the organism has infected the joint.
b. Organisms with a tendency to infect joints hematogenously include *Mycoplasma* spp. in goats[58] and *Borrelia burgdorferi* in acute infections.[59,60]
c. Other microscopically detectable agents that have caused joint infections include other bacteria,[40,61–63] fungi (e.g., *Histoplasma*, *Candida*, *Aspergillus*, *Talaromyces*),[64–66] and protozoa (e.g., *Leishmania donovani*).[67,68]

2. Cytoplasmic inclusions suggestive of immune-mediated disease
a. A LE cell is a neutrophil that contains a single large homogeneous inclusion of phagocytized nuclear material (histones) complexed with autoantibody.[69,70] LE cells have a characteristic microscopic appearance in synovial fluid smears stained with routine Romanowsky-type stain (Figure 20.3A) and are supportive of immune-mediated arthritis when present with other typical findings. LE cells may also be generated in blood samples from some dogs with immune-mediated arthritis via the LE test (see Selected Ancillary Analyses for Synovial Effusions below).[71]
b. Neutrophils containing low to moderate numbers of variously sized (typically < 2 µm wide), purplish cytoplasmic inclusions with Romanowsky staining have been associated with immune-mediated joint disease (Figure 20.3). They are typically present when LE cells are detected,[72] and they are often present without LE cells. They also form in some positive LE test preparations. The inclusions are thought to contain nuclear material and antibodies to nuclear epitopes, but their composition has not been established in veterinary species.
c. In veterinary medicine, the inclusion-body neutrophils described above are often referred to as *ragocytes* (grape cells, from the French *cellules à grains de raisin*, which

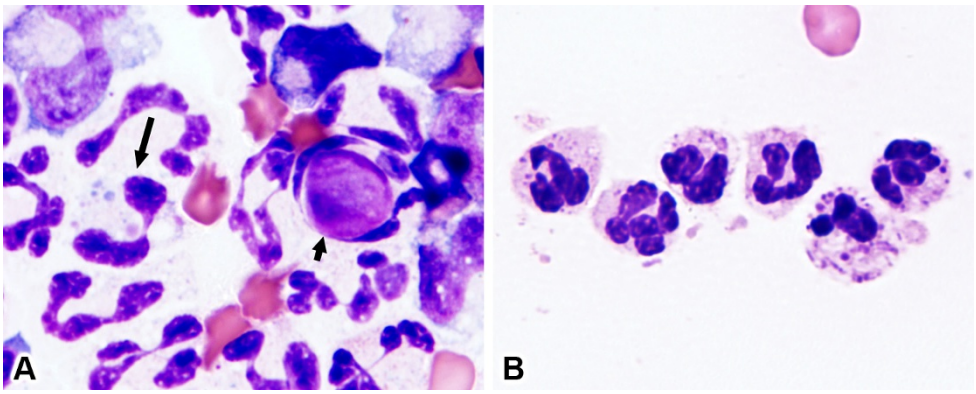


Figure 20.3. A. LE cell (*short arrow*) in a cytocentrifuge preparation of hyaluronidase-treated synovial fluid from a dog with IMPA. The LE cell is a neutrophil with a purplish inclusion that lacks a chromatin pattern and displaces the nucleus peripherally. Other neutrophils contain presumed immune complexes (*long arrow*). B. Neutrophils with irregularly sized and shaped blue to purple inclusions (presumed immune complexes) in a direct smear of synovial fluid from a dog with IMPA. Compare to the more regular bacterial cocci in Figure 20.4. Cell rowing and incomplete flattening reflect viscosity. Wright stain, 100× objective.

does not mean raisins or raisin seeds as sometimes translated).[73] However, in human medicine, this term is used to refer to synovial fluid WBCs, mostly neutrophils, containing refractile granules when viewed in fresh, unstained, wet preparations of synovial fluid. These have also been called inclusion-body cells, and the granules reportedly do not stain with Wright or Giemsa stains.[74] When detected in human patients with rheumatoid arthritis, they have been more specifically called "R.A. cells" for rheumatoid arthritis cells, and their content was shown to include DNA and RF immune complexes. In veterinary medicine, unstained wet preparations of synovial fluid are not routinely assessed for ragocytes.

d. A tart cell, named after a patient (Tart) whose bone marrow contained many such cells, is a macrophage containing a phagocytized nucleus with discernible chromatin structure.[70,75] This appearance is common to macrophages that have engulfed nucleated cells in a wide variety of states unassociated with antinuclear antibodies, e.g., inflammation, so it is not indicative of immune-mediated disease. Tart cells should therefore be recognized so that they are not mistakenly identified as LE cells. Similarly, neutrophils may contain inclusions of nonhomogeneous nuclear material that does not meet visual criteria for LE cells.[75]

3. Extracellular and intracellular crystals likely to be calcium pyrophosphate have been rarely reported in synovial fluid of dogs with neutrophilic arthropathies,[76,77] but they may have been secondary rather than a primary cause of the arthropathies. Extracellular brown needle-like crystals with typical birefringence of monosodium urate have been reported in a few dogs and a cat, all with lameness attributed to gout.[78]
4. Injected material, such as silicone,[79] may be rarely detected extracellularly or within phagocytes.
5. Extracellular nonbirefringent black metallic material released from a worn implant can be present with postarthroplasty metallosis.[80]

IV. Total nucleated cell concentration (TNCC): Measurement of TNCC is useful in differentiating causes of synovial effusions when combined with a microscopic differential count of nucleated cells.

A. Automated hematology analyzers may be used if there is enough sample volume, there are no clots, and the sample is treated with hyaluronidase to reduce viscosity. Lack of a "gold standard" reference method limits assessment of analytical performance, but comparisons of automated and hemocytometer TNCCs in canine, equine, and human synovial fluid suggest that automated methods perform reasonably well for routine diagnostic purposes (for TNCC, not for concentrations of each cell type).[12,48,81–83]

B. Microscopic methods

1. TNCC can be determined by using a hemocytometer (Figures 2.3 and 21.2), the standard method before the advent of automated methods. Hyaluronidase treatment may be needed. Dilutions, if necessary, should be done with saline. This method is more labor-intensive, more time-consuming, and less precise than automated methods, but it may allow counting of individual cells within clusters and counting of large cells that might not be included via automated analyzers. It may be preferred with septic joints to limit introduction of bacteria into an analyzer.
2. Cell concentrations can be crudely estimated based on the number of cells observed in an average microscopic field of an appropriate smear area using a given objective and ocular field diameter. This is difficult with routinely submitted direct smears because of varied smear thicknesses and therefore cell density per microscopic field. Estimates may be improved by using smears made after hyaluronidase treatment to reduce viscosity, or

perhaps by a standardized smearing technique. However, estimates generated by smear estimates are error-prone, even with meticulously standardized smear preparation and cell enumeration procedures (see the preceding Section III.A.2),[56] so they should not be considered universally reliable.[84]

C. Increased TNCC is evidence of disease, though disease may be present without it. Interpretation depends on the type(s) of cells that are increased, as well as other findings. Blood contamination can contribute to an increased TNCC, but the effect likely varies with the patient's hemogram and specifics of the vascular trauma. In an experimental spiking of equine synovial fluid with autologous blood,[85] effects on TNCC could be calculated, but the value of such calculations with iatrogenic blood contamination during arthrocentesis is not known. Interpretation is largely subjective.

V. Refractometric estimate of [total protein] ([TP_{ref}])

A. Refractometry is a common method of estimating [TP]. The serum or plasma [TP_{ref}] scale of clinical refractometers can be used to estimate a synovial fluid's [TP] because the refractivity of total solids of synovial fluid is similar to that of plasma.[86]

B. Synovial fluid does not need to be centrifuged for this measurement because the fluid's refractivity is dependent only on dissolved solutes; suspended particles or cells do not alter the fluid's refractive index. If a sample was placed into a tube with K_2EDTA, the anticoagulant fluid solutes (EDTA, K^+, or other contents) may increase the refractive index when there is a small volume of synovial fluid (e.g., < 0.5 mL in a 3 mL tube).[46]

C. As described in Chapter 7, abnormally high concentrations of solutes (e.g., glucose, urea, electrolytes) falsely elevate the [TP_{ref}].

D. Increased [TP_{ref}] is expected with substantial hemorrhage and with inflammatory diseases that cause local increases in vascular permeability, though hypoproteinemia would reduce the magnitude of change. In an experimental spiking of equine synovial fluid with autologous blood,[85] effects on [TP_{ref}] could be calculated, but the value of such calculations with iatrogenic blood contamination during arthrocentesis is not known.

E. [TP_{ref}] may be reduced after flushing joints with crystalloid solutions.

VI. Refractometric estimate of specific gravity

A. The specific gravity scale in clinical refractometers is calibrated for urine and not proteinaceous synovial fluid. The [TP_{ref}] can be converted to an appropriate specific gravity value using conversion tables for plasma.[86]

B. The converted specific gravity value has no clinical relevance because that physical property does not relate to physiologic or pathologic mechanisms. Alterations in the converted specific gravity values are reflected by proportional alterations in [TP_{ref}].

VII. Hematocrit or [RBC]: The RBC component may be determined in blood-tinged (pink or red) samples by measuring the spun hematocrit of the fluid or by enumerating RBCs in an automated hematology analyzer or hemocytometer. Results below the analytical ranges of these methods should not be reported or interpreted.

SELECTED ANCILLARY ANALYSES FOR SYNOVIAL EFFUSIONS

Ancillary testing may be indicated to further characterize synovial fluid abnormalities.

I. Mucin clot test

A. Although unlikely to provide clinically useful information beyond that obtained by microscopic examination, this semiquantitative test can be used to assess quantity and quality of hyaluronan–protein complexes.
B. Laboratories are inconsistent in sample requirements for the mucin clot test, some not accepting synovial fluid in EDTA. EDTA was shown to decrease mucin clot strength in the presence of metals (including via use of a stainless steel viscometer) or certain added reducing agents, but it did not affect results in a glass system.[87] EDTA samples are not expected to be problematic using current methods.
C. Synovial fluid not treated with hyaluronidase (may be obtained directly from a collection needle and syringe) is added to a solution of dilute acetic acid. Protocols have varied, some entailing addition of 1–2 drops of synovial fluid to about 4 mL of 1 % (weight/volume) acetic acid,[5] while others have described the addition of 1 drop of synovial fluid to 3 drops of 1 % acetic acid or a few drops to 10 mL of 5 % acetic acid.
D. Precipitation of hyaluronan and protein forms a mucin clot (not a fibrin clot), which is graded using agitation to assess clot strength as follows:
    1. Good: large, tight mucin clot surrounded by clear fluid
    2. Fair: soft mucin clot in a turbid fluid
    3. Poor: friable mucin clot in a cloudy fluid
    4. Very poor or absent: flocculent material in a cloudy fluid
E. Good mucin clots are expected in health; decreased mucin clot formation may occur with the following.
    1. Degraded or decreased hyaluronan–protein complexes from bacterial or cellular enzymes including hyaluronidase (inflammation ± infection)
    2. Decreased concentration of hyaluronan–protein complexes from dilution (e.g., substantial hemorrhage, surgical flushing, marked fluid extravasation, decreased lymphatic drainage) or decreased formation by synovium
    3. Exposure to hyaluronidase or EDTA in the presence of metal

II. Antinuclear antibody (ANA) test
A. Purpose: Detection of antinuclear antibodies is used to support the diagnosis of autoimmune disorders, particularly SLE, but also several other disorders that are recognized primarily in people. *Antinuclear antibodies* are autoantibodies directed against double-stranded DNA, histones, or RNA-binding proteins and their complexes with RNA (ribonucleoprotein). Some nuclear antigens targeted by human ANAs are also targeted by ANAs in domestic species, but other antigens of domestic mammals are different and uncharacterized.[88–90]
B. Analytical methods[91–93]
    1. Most commonly, serial dilutions of patient serum are incubated with monolayers of rat liver or human epithelial type 2 (HEp-2) cells that are then washed and incubated with species-specific fluorochrome-labeled anti-IgG antibodies. Compared to rat liver, HEp-2 cells have less nonspecific reactivity with canine serum and yield clearer fluorescence patterns.[94] Fluorescent microscopy is used to detect the greatest dilution at which serum yields positive fluorescence as well as the pattern of fluorescence; the pattern relates to the distribution of reactive epitopes. Major patterns recognized in veterinary medicine are (i) homogeneous (diffuse), with positive chromosomal fluorescence of mitotic cells, and (ii) speckled, with negative chromosomal fluorescence of mitotic cells, but other patterns occur. Sources of variation in results include the type of cells used and their autoantibody profiles, cell fixation conditions, reagent antibody specificity, microscope quality, and microscopist training.

2. Other immunoassay methods developed to reduce subjectivity and labor while characterizing specific antibody targets include ELISAs, chemiluminescent assays, line immunoblot assays, and bead assays in which one or more nuclear antigens is adsorbed to a substrate and incubated with patient serum or plasma. Smears containing *Crithidia luciliae* trypanosomes are also used to assess antibodies to double-stranded DNA, which is present in their large kinetoplasts. Results of all these assays vary with the type and quantity of the antigens as well as with the reagents, assay conditions, and signal detection; the number of antigens assessed by these methods is generally restricted compared to indirect immunofluorescence methods using whole cells.

C. Interpretation: Because of method differences, the titer level considered to reflect positivity varies among laboratories. Negative titers do not support SLE; positive titers support SLE in the presence of appropriate clinical signs and when other neoplastic, infectious (e.g., ehrlichiosis, bartonellosis, leishmaniasis), or noninfectious inflammatory diseases are excluded. In one study, ANAs were detected in 45 % of dogs with seroreactivity to multiple vector-borne organisms,[95] but in another study, dogs with leishmaniasis and arthritis were all ANA-negative.[96] Even in the absence of clinical or laboratory evidence of disease, ANA results may be positive. Strongly positive ANA results were reported in 20 % of apparently healthy client-owned cats, with another 10 % having weak positive results.[97] There is little information regarding clinical significance of different autoantibody profiles in veterinary medicine.
   1. In dogs, homogeneous patterns have been associated with antibodies to nucleosomes or double-stranded DNA and a greater likelihood of multiorgan disease (potentially SLE), while antibodies to ribonucleoproteins or RNA-binding proteins are associated with a speckled pattern and polyarthritis but fewer features of SLE.[88,93]
   2. In one study using an assay to detect anti-histone antibodies, high titers supported a diagnosis of SLE (the diagnosis in 11 of 12 dogs with the highest titers).[98]

III. Rheumatoid factor (RF) test

A. Purpose: Species-specific RF tests may be used to investigate or support the diagnosis of rheumatoid arthritis–like conditions. *Rheumatoid factor* is the term used for autoantibodies to the Fc portion of IgG. Most are IgM, but they may also be IgA and IgG. They may promote disease by forming immune complexes in joints. Low concentrations of low-affinity RFs are present in health, increasing with age; they promote complement fixation on immune complexes, immune complex clearance, and uptake of immune complexes for antigen presentation.[99] Increased titers of higher-affinity RFs occur in 70–80 % of human patients with rheumatoid arthritis, so titers are measured to help diagnose, classify, and even predict the condition.

B. Analytical methods
   1. In human medicine, most serologic tests for RF are now ELISAs or nephelometric assays, the latter using fixed amounts of heat-aggregated IgG, chemically cross-linked IgG, or IgG-coated latex beads to react with patient serum RF and form immune complexes detectable by light scatter. Factors affecting these tests have been summarized elsewhere.[100]
   2. Assays developed to detect IgM RF in domestic mammals (mostly dogs) include hemagglutination assays, latex bead agglutination assays, ELISAs, gel precipitation assays, and radioimmunoassays. Results have varied substantially, in part because of sample differences and in part because of many methodologic differences.[101–103] The major sample difference is whether or not the serum is heated in a way that inactivates

complement. Such heating reduces positivity in samples from many dogs, particularly healthy ones; this may be because of a desirable reduction in complement-mediated binding of IgM to assay IgG or because of an undesirable heat-induced formation of IgG aggregates that bind sample RF and reduce its detection. A major assay difference is whether the target IgG has been sufficiently aggregated or bound to an antigen such that RF binding occurs.

C. Interpretation: The most favorable results suggest that most healthy dogs are seronegative and some dogs with suspected rheumatoid arthritis are seropositive, but dogs with SLE, idiopathic polyarthritis, and a variety of other nonimmune conditions including OA may be seropositive as well.[102,103] Results based on assays that did not use heat-treated serum samples and/or complexed IgG had unclear analytical specificity for RF and appeared to lack diagnostic utility for diagnosing rheumatoid arthritis or immune-mediated disease.[104,105] Recent reports of analytical or diagnostic validation for available assays were not found.

D. In human medicine, ELISAs for anti-(citrullinated protein) antibodies (IgM, IgA, or IgG) are also widely used to diagnose rheumatoid arthritis, and they have greater diagnostic specificity than RF, with similar diagnostic sensitivity. Citrulline is an amino acid that forms from the amino acid arginine by deimination (citrullination). In rheumatoid arthritis, dysregulated enzymatic posttranslational citrullination of arginine in proteins such as fibrinogen, α-enolase, vimentin, and type II collagen lead to the formation of anti-(citrullinated protein) antibodies. A synthetic library of antigens (cyclic citrullinated peptides, CCPs) has been developed to yield ELISAs with optimal diagnostic value for the detection of so-called anti-CCP antibodies in people.[100] Anti-(citrullinated fibrinogen) antibodies have been assessed in healthy horses but published details of the assay's analytical validation are lacking.[106]

IV. Lupus erythematosus (LE) cell test

A. Purpose: LE cell testing may be done to support a diagnosis of SLE, but testing is uncommon, in part because it is labor-intensive, it has low diagnostic sensitivity, and it involves subjectivity (differentiation of LE cells from tart cells). The LE cell test has been largely replaced by ANA testing.

B. Analytical methods: Various tube, slide, and capillary methods have been developed to incubate patient serum or plasma with blood (or marrow) WBCs from the patient or another individual of the same species after the WBCs have been traumatized to expose nuclear epitopes.[107,108] Cell and nuclear damage is inflicted by forcing clotted blood through a mesh or forcefully stirring cells (e.g., with a needle). If antinuclear antibodies are present in the patient serum or plasma, they bind to exposed nuclear epitopes and promote phagocytosis of the immune complexes to form LE cells. LE cells or rosettes of neutrophils around extracellular immune complexes are identified microscopically in Romanowsky-stained smears of the incubated cells. Incubation has been done at room temperature or 37 °C, the latter for less time. The number of detectable LE cells can be markedly influenced by the method and the quality of the smears.[107]

C. Interpretation: Detection of definitive LE cells is a positive test that supports the presence of antinuclear antibodies and therefore, potentially, an autoimmune disease including SLE. Rosette formation supports further evaluation for LE cells if not already detected.

V. Microbiological assessment

A. Microbial culture of synovial fluid may be indicated if infection in the joint is suspected based on (i) history, such as an invasive procedure or trauma involving the joint, (ii) results

of routine analysis of synovial fluid, such as a high concentration of neutrophils with no other apparent cause, particularly in a single joint or when polyarthritis occurs with clinical evidence of a systemic bacterial infection (e.g., a newborn calf with omphalophlebitis), or (iii) to further characterize an infection that has already been detected based on microscopic examination of the fluid. Polymerase chain reaction amplification might also be used to help detect or characterize certain organisms.

B. Culture of synovial fluid from an infected joint may yield false-negative or false-positive (contamination) results. In a study of experimental *Staphylococcus intermedius* joint infection in dogs, culture of synovial membranes was not more sensitive than culture of synovial fluid in culturettes or blood culture medium.[109] A study in horses found that culturing synovial fluid resulted in bacterial growth more often than did culturing synovial membrane.[110]

C. Current or very recent antimicrobial therapy could potentially cause false-negative culture results, especially if the amount of drug in the synovial fluid is greater than the minimum inhibitory concentration.

COMMENTS ABOUT SPECIFIC TYPES OF ARTHROPATHIES

I. Neutrophilic arthropathies: Neutrophils are the predominant nucleated cell type in most effusions caused by joint infection or immune-mediated disease. If neutrophilic inflammation is the main abnormality identified based on routine testing, then further evidence of infection or immune-mediated disease should be sought.

A. Joint infections (septic arthritis) (see Section III.D.1 of Routine Analysis of Synovial Fluid, above, for more information about some specific infectious agents)

1. Bacterial: Bacteria are the most common cause of infectious arthritis, reaching joints hematogenously or by direct penetration. Detection of intracellular bacteria on microscopic examination of a freshly made smear of synovial fluid is diagnostic for a bacterial infection (Figure 20.4A), but lack of detection does not exclude an infection.

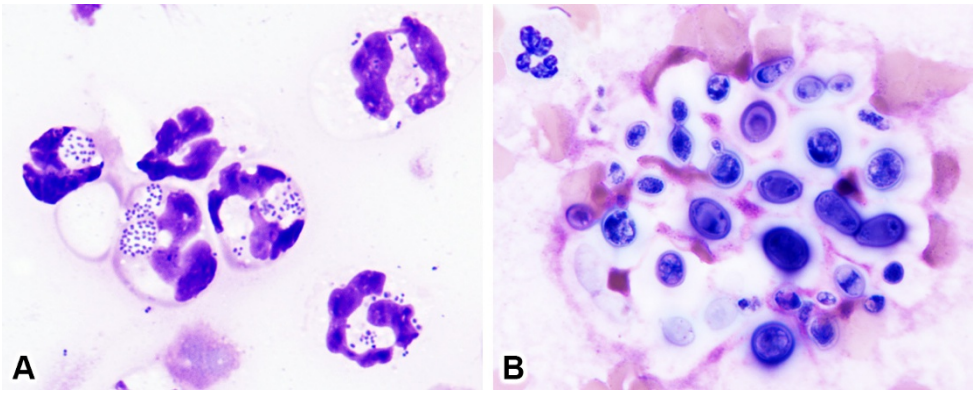


Figure 20.4. A. Degenerate neutrophils containing small bacterial cocci (streptococci were cultured) in the feathered edge of a direct smear of synovial fluid from the right stifle of a dog with acute right hindlimb lameness, 100× objective. B. Cluster of *Cryptococcus* sp. and a nondegenerate neutrophil in a direct smear of synovial fluid from a dog. Note prominent nonstaining capsules and cell walls, and narrow-based budding of the yeast, 50× objective. Wright stain.

a. Bacteria that have undergone phagocytosis are usually in neutrophils. Some bacteria (e.g., *Ehrlichia ewingii*) may be present in neutrophils that became infected outside the joint and then carried the organisms into the joint. Others, such as *Mycoplasma* and *Borrelia* (in acute infections) are expected to be extracellular, and *Mycoplasma* can be difficult to recognize.
b. Bacterial contamination of a sample may mimic infection. Phagocytosis of organisms by neutrophils or macrophages may occur in vitro if there is a delay between when the sample was placed into a tube and when the slide was prepared for microscopic examination. When bacterial rods or cocci that typically undergo phagocytosis are present only extracellularly and in the absence of increased concentrations of inflammatory cells, contamination of the sample or stain is strongly suggested.
c. Neutrophils may appear degenerate in a septic environment, developing swollen nuclei and cytoplasmic vacuoles. When present in freshly made cytologic preparations, degenerate neutrophils indicate that further effort should be taken to identify organisms microscopically or by other special tests. However, infections may be present when neutrophils are nondegenerate, and in vitro deterioration of neutrophils can mimic in vivo degenerative change.

2. Viral: Feline calicivirus infection has been shown experimentally to infect joints and cause acute clinical arthritis.[31] Synovial fluid changes were not well characterized. A naturally occurring arthropathy induced by calicivirus was suspected in a pyretic kitten with upper respiratory signs.[111] Synovial fluid from the affected joint appeared to contain a virulent calicivirus, and the fluid had a very high TNCC with a neutrophilic component to the inflammation, although neutrophils were a minority population. Polyarthritis was suspected to be caused by calicivirus in three cats with symptomatic joints containing caliciviral RNA or antigens, but synovial fluid findings were not well described, and a cause-and-effect relationship was not proven.[112]
3. Other (rare): Fungi (e.g., *Blastomyces*, *Histoplasma*, *Cryptococcus*) or protozoa (e.g., *Leishmania*, *Trypanosoma*) may be detected microscopically in synovial fluid samples, typically as part of systemic infections (Figure 20.4B). *Histoplasma* and *Leishmania* organisms are usually within macrophages or extracellular. *Blastomyces* and *Cryptococcus* yeast are commonly extracellular, but they may be within macrophages, and *Trypanosoma* trypomastigotes are extracellular. When only extracellular fungi are present, particularly in the absence of an inflammatory response, contamination of the sample, the stain, or the slide should be considered.

B. Arthropathies known or suspected to be immune mediated (usually polyarticular): These sterile neutrophilic arthropathies that generally respond to immunosuppressive therapy cannot be differentiated by synovial fluid analysis alone but have been subdivided into several conditions and syndromes based on other laboratory and clinical information including age, breed, presence or absence of extra-articular disease, potential initiating lesions such as infections or neoplasms, presence or absence of radiographically apparent erosive changes, serologic findings (ANA titers, titers to infectious agents such as *Borrelia*), and histologic or gross pathologic findings. They are recognized most often in dogs, less often in cats and horses, and rarely in other species.
1. Immune-mediated polyarthritis (IMPA): IMPA is often idiopathic and unassociated with other clinical abnormalities, but it has also been associated with remote infections, previous infections, gastrointestinal inflammatory disease, and neoplasms.[34,35,37,113–119]
a. A diagnosis of IMPA requires detection of neutrophilic inflammation in multiple joints and exclusion of other causes, including joint sepsis. Neutrophils containing

inclusions suggestive of immune complex material (Figure 20.3) strongly suggest that an effusion is immune-mediated, but these are not detected in most cases of immune-mediated joint disease.

b. Veterinarians treating animals with IMPA often obtain repeated samples of synovial fluid to monitor response to therapy. A study in healthy dogs found no increase in neutrophil percentages or estimated neutrophil concentrations 3 wk after each of four arthrocentesis samples taken at 3-wk intervals.[120]

2. Systemic lupus erythematosus (SLE) or SLE-like syndrome: Neutrophilic arthropathy typical of IMPA accompanies other clinical and laboratory abnormalities that support a diagnosis of SLE. In people, SLE is a heterogeneous waxing and waning systemic autoimmune disease that most commonly involves mucocutaneous lesions, arthritis, nephritis, hematologic disorders, or fever; ANAs and anti-phospholipid antibodies are common, but antibodies to other targets form as well. The initiating cause is unknown. Differences in the human and canine conditions have been discussed.[121] LE cells and/or neutrophils containing inclusions suggestive of immune-complex material (Figure 20.3) may be present in synovial fluid.[72,122]
3. Rheumatoid arthritis
   a. Human rheumatoid arthritis is a progressive, systemic, heterogeneous, immune-mediated disease associated with autoantibodies and characterized primarily by a somewhat symmetrical polyarthritis and destruction of synovial joints.[36] The initiating cause is unknown. Identification and naming of this disorder have a complicated history reflecting a resemblance to the polyarthritis of rheumatic fever, a disorder caused by streptococcal infections; *rheumatoid* stems from the Greek *rheuma,* which refers to that which flows, likely applied to describe what was considered to be a flow of "the humors into the joints."[123]
   b. In veterinary medicine, some cases of erosive IMPA, which is much less common than nonerosive IMPA, have been considered rheumatoid arthritis based on similarities to the human condition and use of human classification criteria.[124–127,128] Diagnostic criteria for human rheumatoid arthritis were developed in the 1950s, modified into classification criteria in 1987,[129] and updated in 2010 to help classify early rheumatoid arthritis.[130] Although these criteria, which do not include arthrocentesis or synovial fluid analysis, have been used for the diagnosis of human and veterinary rheumatoid arthritis, they were intended only for classification of rheumatoid arthritis once other conditions with a similar clinical presentation were excluded. Whether veterinary conditions diagnosed as rheumatoid arthritis have been rheumatoid arthritis or rheumatoid arthritis–like (idiopathic erosive polyarthritis) is unclear.
4. Other: Clinical subclassifications of immune-mediated neutrophilic arthropathies include (i) syndromes with other concurrent lesions that are thought to be immune mediated (e.g., myositis, meningitis),[35,125,131,132] (ii) nonerosive polyarthritis in juvenile Akitas (some with meningitis),[132] (iii) erosive polyarthritis in young greyhounds,[133,134] and (iv) vaccination and drug reactions.[135,136]

C. Trauma: External or internal physical damage to a joint may induce hemorrhage and inflammation, the nature of which is generally mild to moderate and varies from macrophagic to neutrophilic or mixed. In a retrospective study of arthritic cattle, the 26 considered to have noninfectious traumatic arthritis (criteria not provided) had median TNCC and neutrophil concentration of 1200/μL and 200/μL, respectively, with maximum values of 40,100/μL and 38,100/μL, respectively.[137] The remaining cells were classified only as "mononuclear cells." Traumatic arthritis was carefully characterized in two other cattle that had posterior paresis

and self-induced trauma from repeated attempts to stand.[138] TNCCs were mildly to moderately increased (up to 12,600/μL) and lymphocytes predominated, although neutrophil concentrations were mildly to moderately increased as well. Lipohemarthrosis was evidenced by xanthochromia, RBCs, and lipid globules that were most evident when they rose to the fluid surface of the sample upon standing or centrifugation. Regarding horses, it is the authors' clinical impression that mild to moderate sterile neutrophilic inflammation occurs in joints adjacent to inflammatory lesions such as traumatic wounds, but documentation was not found.

D. Joint injections / foreign material: Various substances have been injected into the joints of animals for therapeutic (e.g., antibiotics, balanced crystalloid solutions for lavage, lubricants) or research purposes. Injecting any material into a joint is likely to elicit some inflammatory response, which will vary in nature, severity, and duration, depending on the specific stimulus. Hyaluronic acid injected into fetlocks of healthy standardbred horses decreased synovial fluid viscosity and caused mild increases in TNCC, [TP_{ref}], [RBC], and joint effusion scores.[139] Even injection of lactated Ringer's solution into the joints of clinically healthy horses caused increased TNCC (as high as 20,000/μL)[140,141] and, in some cases, increased percentage of neutrophils, both lasting several days.[141,142] Arthrocentesis alone had similar, but milder, effects.[142]

E. Pancreatitis, panniculitis, and polyarthritis syndrome: This constellation of findings has been rarely reported in dogs and people with benign or malignant pancreatic disease and markedly increased serum lipase activities. Although synovial fluid findings have not been well characterized in dogs, neutrophilic inflammation is typical of samples from affected people and has been detected in synovial fluid of a few dogs with arthritis and concurrent pancreatitis or pancreatic neoplasms.[143,144] Inflammation is thought to be secondary to lipolysis of periarticular and interosseous adipose tissue.

F. Shar pei autoinflammatory disease (swollen-hock syndrome, familial amyloidosis, shar pei fever):[145,146] Affected shar peis develop polyarthritis or other inflammatory lesions because of what is thought to be an *autoinflammatory disease*, i.e., a sterile hyperinflammatory state caused by dysregulation of the innate immune system, not by the acquired immune system as for the immune-mediated polyarthritides discussed above.

II. Macrophagic arthropathies: Because macrophages are the predominant nucleated cell type in synovial fluid in health, recognizing a macrophagic arthropathy requires a clear increase in TNCC or clear abnormalities in the appearance of macrophages (e.g., increased phagocytic activity) in addition to macrophage predominance. As noted previously, low-grade macrophagic inflammation may be present when synovial fluid analysis cannot definitively reveal it or differentiate it from health, so it is often not clear from routine analysis whether a sample is from a healthy or diseased joint.

A. Osteoarthritis: TNCC is typically within reference limit or mildly increased.

1. Tissue damage in OA can incite an inflammatory response favoring recruitment of monocytes/macrophages and resulting in an increase in synovial fluid macrophage concentration. Fluids from joints with OA have been found to have increased expression of proinflammatory molecules,[147–152] but neutrophils and lymphocytes are typically absent or present in low concentrations.
2. It is plausible that OA could elicit increased cytoplasmic vacuolation in macrophages, but documentation is lacking. In a study of nine synovial fluid samples from clinically healthy adult dogs, the majority of cells classified as monocytes/macrophages had moderate to marked vacuolation (the criteria for determining severity of vacuolation

were not provided).[11] Macrophage vacuolation may occur in vitro with delays to sample analysis, so caution should be exercised in the interpretation of cell vacuolation.

B. Foreign material: Macrophagic inflammation associated with intra- and extracellular globules of silicone was present in synovial fluid from a horse that had recently received an intra-articular injection of silicone; another horse with intra-articular silicone had low-grade neutrophilic and macrophagic inflammation.[79]

C. Repeated arthrocentesis: A study of repeated sampling of synovial fluid from healthy dogs at 3-wk intervals found some dogs to develop mildly increased macrophage concentrations based on microscopic differential counts and microscopic estimation of cell concentrations.[120]

III. Lymphocytic arthropathies: Increased concentrations of lymphocytes may be present in synovial fluid with neutrophilic or macrophagic arthropathies, but effusions in which lymphocytes predominate or account for a high proportion of nucleated cells are rare. When an increased concentration of nonneoplastic lymphocytes is present, it reflects a lymphocytic inflammatory response, but the cause is typically unknown.

A. An increase in TNCC and synovial fluid T lymphocytes occurs in goats infected with caprine arthritis encephalitis virus.[153,154]

B. In a kitten with evidence of a calicivirus-induced arthropathy, the TNCC in a stifle joint was markedly increased and 80% of the nucleated cells were reportedly "small mononuclear cells" and therefore possibly lymphocytes (other cell types can appear smaller than usual and often lymphoid when cells do not spread well, especially in thick smear preparations).[111]

C. It is noteworthy that synovial fluid findings do not mirror synovial membrane findings; the presence of lymphoplasmacytic synovitis in dogs with IMPA or ruptured cranial cruciate ligaments is typically not associated with lymphocyte-rich synovial effusions.[38,155]

D. Importantly, macrophages and neutrophils are easily mistaken for lymphocytes when they are "balled up" in thick synovial fluid preparations, so fluids may be mistakenly classified.

IV. Eosinophilic arthropathies: Synovial effusions in which eosinophils are a prominent feature are not common in domestic animals. An immune-mediated mechanism is suspected in most cases; other causes include parasitic or nonparasitic hypersensitivity reaction (e.g., to injected foreign material).[156–161]

V. Mixed-inflammatory-cell arthropathies: It is common for samples from patients with various kinds of arthropathies to be composed of more than one type of inflammatory cell. The concentration and proportion of different types of cells probably depends on the nature, magnitude, and duration of the inflammatory stimulus, and perhaps on host variables. The inflammatory process may be labeled "mixed," or it may be placed in one of the other categories based on the cell type that is most increased or supportive of disease (e.g., eosinophils).

VI. Hemorrhagic arthropathies

A. Pathologic hemorrhage may be present in joints with a variety of arthropathies, but when the primary reason for the arthropathy is bleeding, it is considered a hemorrhagic arthropathy. These are caused by trauma or coagulopathic conditions, whereas disorders of primary hemostasis are not associated with hemarthrosis.

B. Mild to marked iatrogenic hemorrhage occurring because of sampling-related trauma may mimic a hemorrhagic arthropathy. The sudden appearance of blood mid-collection would support iatrogenic hemorrhage.
C. The concentration of RBCs in hemorrhagic synovial effusions varies widely depending on severity and timing of bleeding, but the fluid will be red, pink, or brown. It will look like blood when there is acute, severe hemorrhage, and will have a lower [RBC] and appear less red and perhaps brownish as time passes. The following microscopic findings may be present to support pathologic hemorrhage.
    1. Macrophages containing RBCs
        a. Engulfed RBCs in a smear made from a freshly obtained sample is definitive evidence of hemorrhage prior to sample acquisition, but the hemorrhage may be a major or minor component of the effusion.
        b. A delay between when the sample was placed into a tube and when the slide was prepared for microscopic examination can cause in vitro phagocytosis of RBCs obtained incidentally during sampling.
    2. If more time has elapsed since bleeding occurred (probably days), RBC breakdown products may be present:
        a. Macrophages containing hemosiderin (siderophages)
        b. Hematoidin crystals (bilirubin) within macrophages or extracellularly

VII. Neoplastic arthropathies: Rarely, neoplastic cells from a variety of primary or metastatic neoplasms including carcinomas, soft tissue sarcomas, and lymphoma have been reported in synovial fluid.[39,162–164] Inflammation or hemorrhage may also be present. In the absence of neoplastic cells, laboratory classification would be based on any major pathologic process (e.g., inflammation or hemorrhage), but clinically, the arthropathy might be classified as neoplastic based on other findings.

References

A complete set of references is available online. Please use the below link to access the References files.

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Chapter 21

CEREBROSPINAL FLUID

Michael A. Scott and Steven L. Stockham

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Table 21.1. Abbreviations and symbols in this chapter	
[x]	Concentration of x (x = analyte)
CNS	Central nervous system
CSF	Cerebrospinal fluid
EDTA	Ethylenediaminetetraacetic acid
RBC	Red blood cell (erythrocyte)
RI	Reference interval
TNCC	Total nucleated cell concentration
URL	Upper reference limit
WBC	White blood cell (leukocyte)

Although routine analysis of CSF is a valuable tool to assess patients with known or potential neurologic disease, it brings many challenges (see Physiologic Processes and General Concepts IV. Challenges of CSF Analysis and Interpretation). Optimal analysis requires methods, equipment, and analytical expertise that are typically available only at referral laboratories. And, unfortunately, delivery of CSF samples to such laboratories promotes preanalytical errors related to sample processing and delayed analysis, errors that may be compounded by sample collection challenges. Furthermore, even in the absence of such preanalytical and analytical errors, CSF results provide limited specificity for particular disorders, and appropriate interpretation requires thorough clinical knowledge of the patient and sometimes specific expertise in neurology. Despite these challenges, interested individuals can learn to generate valuable in-house information via a hemocytometer for cell concentrations, gravity sedimentation for cytologic preparations, and, to a limited degree, semiquantitative protein tests.

PHYSIOLOGIC PROCESSES AND GENERAL CONCEPTS

CSF bathes and cushions the CNS, protecting it from its own weight and from physical trauma. As a circulating fluid, it also provides an appropriate environment for brain functions, a source of H_2O to shift into CNS tissue to minimize the ill effects of dehydration, and a medium for molecular transport, circulation of disease-surveilling WBCs, and removal of CNS waste. With CNS disorders, chemical and cellular components of the CSF may be altered. To understand these potential alterations, one must understand CSF dynamics and the barriers surrounding CSF that protect the CNS and maintain a stable ionic environment for neurologic functions despite marked changes in the blood.[1,2]

I. CSF location, production, and dynamics
   A. CSF fills the brain's ventricles and flows through the subarachnoid spaces and the spinal canal. It is bounded by ependymal cells in the ventricular system and spinal canal, and by pia mater and arachnoid mater in the subarachnoid space.
   B. CSF production is continuous, with the entire CSF volume being replaced several times daily. Production occurs in the choroid plexus of each ventricle, with substantial contributions across other ependymal surfaces and via CNS capillaries (Figure 21.1A).[1,2] Although often called an ultrafiltrate of plasma, CSF is produced by passive diffusion and by controlled bidirectional transport such that CSF constituents do not completely parallel plasma and the CNS is protected from certain endogenous and exogenous substances.
   C. CSF circulation and mixing appear to involve (i) directed flow out of the ventricles, in part via the action of ependymal cell cilia, (ii) back and forth movement in response to

respiratory and cardiac-induced pressure changes, and (iii) continuous bidirectional fluid exchange among CNS compartments, including absorption across the blood–brain barrier (Figure 21.1B).

D. The CNS lacks lymphatic vessels to remove excess fluid and wastes. Instead, much of this occurs through unidirectional vesicular transport through arachnoid villi (Figure 21.1C) to blood, a process driven by CSF pressure.[3] This is called *bulk movement*, the passage of all CSF constituents at equal rates regardless of molecular properties. CSF and possibly antigen-presenting cells in subarachnoid spaces move along cranial nerves (e.g., optic, olfactory) through the cribriform plate to reach cervical lymphatics, and along spinal nerve sheaths to reach other lymphatics.[4] Fluid also drains to cervical lymphatics via Virchow–Robin spaces along arterial and arteriolar walls, movement promoted by respiratory and cardiac pulsations.

II. CNS barriers and movement of fluid, solutes, and cells among CNS compartments

A. The *blood–CSF barrier*, the interface between blood and CSF, includes (i) the choroid plexus barrier of the ventricles (Figure 21.1A), (ii) the arachnoid villi barrier in intracranial venous sinuses (Figure 21.1C) and associated with spinal nerve roots, and (iii) the pial microvessel barrier.

B. The blood–brain barrier (Figure 21.1B) actually allows substantial and bidirectional fluxes between blood and interstitial fluid or the Virchow–Robin space (which exchange components with each other) that contribute to the composition of brain interstitial fluid and CSF by additions and subtractions.

1. Water passes through capillary endothelial cells by diffusion, vesicular transport, or in association with solutes such as glucose and Na^+, but passage across the astroglial portion of the barrier relies primarily on astrocyte aquaporin-4 channels.
2. Whereas small solutes such as O_2, CO_2, and NH_4^+ diffuse across the barrier, as do lipophilic molecules including steroid hormones and alcohol, glucose and amino acids cross this barrier by facilitated transport. Large molecules, including most proteins, are mostly excluded from the CNS at this barrier, but some macromolecules pass through by pinocytosis.

C. The *CSF–brain barrier*, the interface between CSF and CNS parenchyma, includes the ependymal cell layer with gap junctions and the outer barrier formed by glial end feet and pia mater. Through this interface, CSF communicates bidirectionally with the Virchow–Robin space and interstitial fluid of the CNS, allowing interstitial fluid and its solutes, including macromolecules, to enter the Virchow–Robin space and drain from there into CSF around penetrating subarachnoid vessels. Osmotic gradients, hydraulic gradients, and transporters, including aquaporins, control fluid exchange, just as they do for the blood–brain and choroid blood–CSF barriers.

D. WBCs leave blood vessels and enter CSF directly via the choroid plexus or indirectly via the Virchow–Robin space of venules, from which they can also enter interstitial fluid. WBCs that are already in CSF can enter interstitial fluid via the Virchow–Robin spaces of venules.

III. CSF composition in health

A. Reliable and applicable reference data on CSF findings in healthy animals are very limited because of variations in sample collection, sample processing, analytical methods, equipment, and cell identification, as well as insufficient numbers of tested individuals. Data from relatively recent reports that included method descriptions are provided as expected features of CSF in health when samples are collected, processed, and analyzed as described

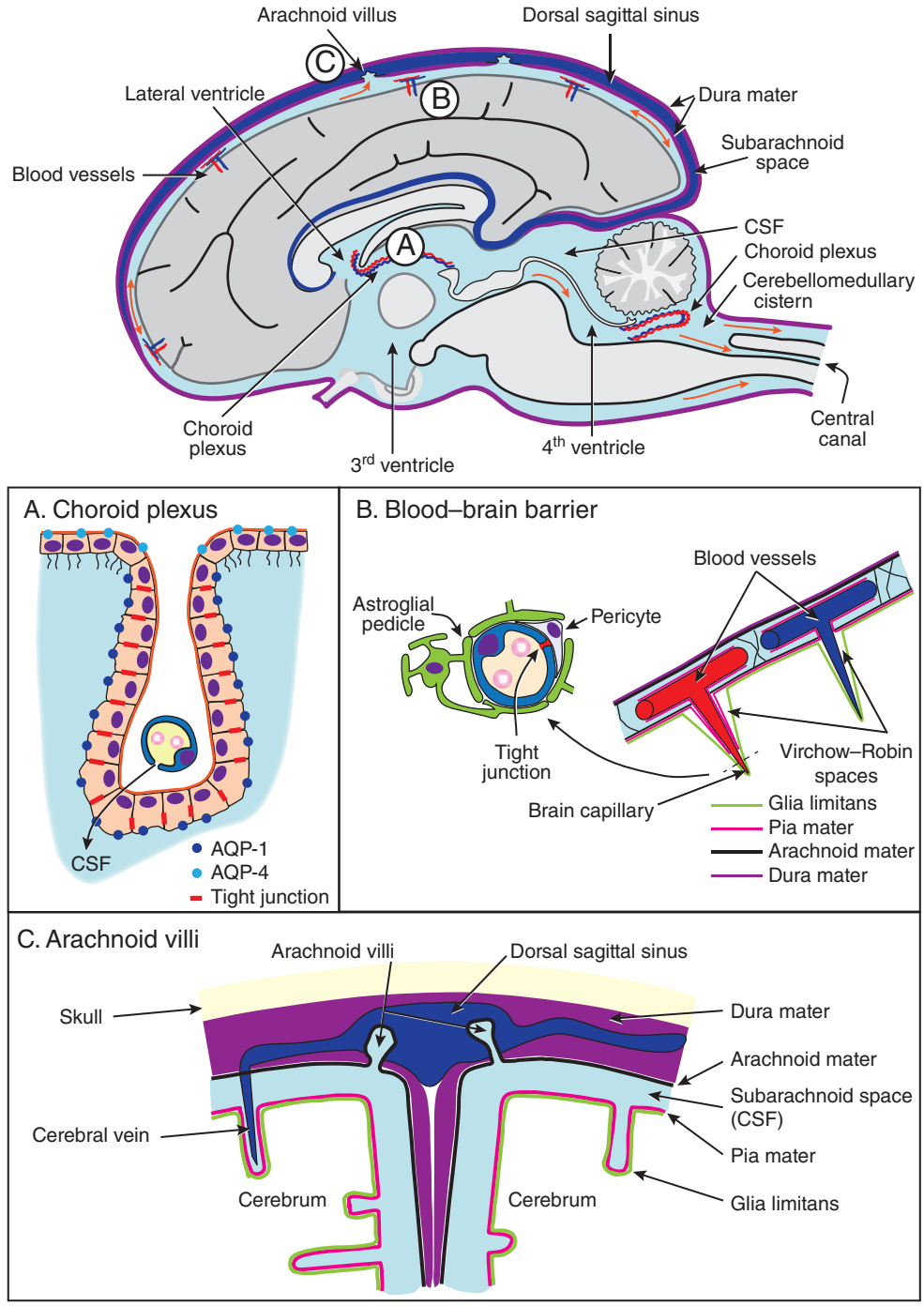


Figure 21.1. Major CNS structural components related to CSF. Much of the CSF is produced by choroid plexuses (A) in the lateral, third, and fourth ventricles of the brain. From there, CSF circulates into the central canal of the spinal cord as well as into the subarachnoid space and cisternae surrounding the brain and spinal cord. CSF bathes and supports the brain and spinal cord, insulates them, and transports nutrients and wastes to and from them. Cells can gain access to CSF via choroid plexuses and Virchow–Robin spaces around brain venules along the blood–brain barrier (B). CSF removal occurs at least in part through arachnoid villi (C) and via drainage along cranial nerves and vessel walls to cervical lymphatics.

(Tables 21.2 and 21.3). Nucleated cell differentials are not provided because of substantial variations in how cells have been classified, the potential for great imprecision due to assessment of few intact cells on many slides, and unknown and likely varied effects of blood contamination.

B. CSF is clear and colorless with the consistency of H_2O. It is 99 % H_2O.

C. It has a very low TNCC (< 10/μL in all domestic mammals), with few lymphocytes and monocytes/macrophages per microliter of fluid. Very few neutrophils, ependymal cells, or leptomeningial cells also may be present.

D. RBCs are presumed to be absent from CSF in health, though they are often present in CSF samples because of iatrogenic hemorrhage during collection.

Figure 21.1. *continued*

A. Choroid plexus: The lateral, third, and fourth ventricles each contain a choroid plexus consisting of villous, frond-like evaginations lined by a single layer of cuboidal epithelial cells that are connected by apical (CSF-facing) tight junctions.
   - Within the villi, permeable capillaries with fenestrated endothelium allow passive diffusion of fluid and solutes from blood to interstitial tissue.
   - Epithelial tight junctions restrict movement of blood constituents to CSF, but the cells take up interstitial H_2O and solutes and secrete them to form CSF in a controlled manner involving ion transporters and AQP-1, a H_2O channel expressed on the apical membrane of choroidal epithelium. In this way, the choroid plexus provides the brain, via CSF, with many needed substances, e.g., neuropeptides, hormones, trace elements, and vitamins.
   - Small amounts of protein are synthesized and secreted into CSF, and a little plasma protein enters CSF by pinocytosis. Active transport also selectively removes certain solutes.
   - Neighboring ventricular ependymal cells are separated from each other by gap junctions and are thought to contribute to CSF production via a mechanism involving basolateral AQP-4 H_2O channels rather than apical AQP-1.

B. Blood–brain barrier: This is the neurovascular interface between the abundant capillaries of the CNS and the surrounding tissue; it restricts and controls CNS contact with blood constituents other than at chemoreceptor and osmoreceptor sites.
   - The *neurovascular unit* forming this neuroencephalic barrier comprises brain capillaries with tight junctions and close association with pericytes, astrocytic foot processes, microglia, and neurons.
   - Although brain capillaries adhere to the *glia limitans*, a lining of astrocytic foot processes surrounding brain tissue, arterioles and venules penetrating brain from the subarachnoid space are surrounded by a space or potential space called the perivascular space or Virchow–Robin space. This links CNS interstitial fluid with blood and CSF. It is enclosed by the glia limitans, pia mater around arterioles, and endothelial basement membrane around venules and capillaries. The pial layer of arterioles becomes more fenestrated and leakier near capillaries, and the perivascular space is occluded at the level of capillaries where the glial basement membrane fuses with the outer vascular membrane. (Note: Some people consider the space between the pia mater and vessels the perivascular space, not a Virchow–Robin space.)
   - Wastes drain from the brain into the perivascular space, and cells from blood move among blood, CSF, and brain compartments through the perivascular spaces.

C. Arachnoid villi: There are protrusions of the arachnoid and CSF-containing subarachnoid space through the dura mater and into venous sinuses around the brain. Arachnoid cells have tight junctions to limit movement of blood constituents to CSF, but they transport CSF across the barrier via vesicles that can be large enough to contain a RBC. They contribute substantially to the movement of CSF H_2O and solutes into blood.

AQP, aquaporin

Table 21.2. Reported features of CSF samples from healthy dogs

	Bailey[b]		Jamison[c]	Bichsel[d]	Galan[e]	Krakowka[f]
Collection site	A-O	Lumbar	A-O	A-O	A-O	A-O
TNCC (/μL)[a]	0–4	0–5	0–2	0–7	0–3	NA
Protein (mg/dL)[a]	14 ± 9	29 ± 11	10–30	NA	12–27	16–42
Method	CBB		PS		Pyro	CBB
Number	31 of each		50	21	11	23

[a] The reported TNCC and [protein] are rounded to the nearest ones unit.
[b] The TNCC was measured by using an unspecified hemocytometer and undiluted CSF; the [protein] reported here is the mean ± 2 SD. A-O samples had RBC concentrations of 0–370/μL, usually 0/μL, with a mean of 30/μL. Five of the lumbar samples had RBC concentrations > 500/μL.[5]
[c] The TNCC was measured by using an unspecified hemocytometer and undiluted CSF.[6]
[d] The TNCC was measured by using a Fuchs–Rosenthal hemocytometer; 0–7/μL is a presumed minimum–maximum. Samples with blood contamination were excluded based on macroscopic and microscopic evaluation (no details provided); 18 of the dogs were beagles.[7]
[e] The TNCC was measured by using a Neubauer hemocytometer and undiluted CSF; all dogs were beagles.[8]
[f] The reported [protein] is for samples with no RBCs; the [protein] in seven dogs with microscopically detected blood contamination was 21–29 mg/dL, and it was 22–51 mg/dL in seven dogs with macroscopically detected blood contamination.[9]
A-O, atlanto-occipital; CBB, Coomassie brilliant blue; NA, not available; PS, Ponceau S; Pyro, pyrogallol red

Table 21.3. Reported features of CSF samples from healthy cats, horses, cattle, and llamas

	Cats[b]	Horses, ponies[c]	Cattle[d]	Llamas[e]
Collection site	A-O	A-O & Lumbar	Lumbar	Lumbar
TNCC (/μL)[a]	0–2	0–6	0–9	0–3
Protein (mg/dL)[a]	6–36	Horses: 5–100	23–66	32–67
		Ponies: 20–105		
Method	PS	TCA	CBB	CBB
Number	33	44	16	17

[a] Reported minimum–maximum TNCC and [protein] are rounded to the nearest ones unit.
[b] TNCC by Bright Line hemocytometer and undiluted CSF. These 33 cats all had a [RBC] < 30/μL. Adding cats with [RBC] > 30/μL (0–3600/μL), the 58 total cats had TNCCs of 0–15/μL and [protein] of 3–46 mg/dL.[10,11]
[c] The TNCC was measured by using a Spencer hemocytometer and undiluted CSF. The basis for provided intervals is not clear but was "based primarily on the mean ± 2 SD." RBC concentrations were 0–2000/μL. TNCC and [RBC] were based on 88 samples, 1 A-O and 1 lumbar from each of 44 animals including 23 or 24 horses (mostly thoroughbred and standardbred but 5 breeds included) and 20 or 21 ponies (18 were Shetland ponies). The [protein] data were based on 44 samples from 22 horses and 42 samples from 21 ponies.[12]
[d] The TNCC was based on unspecified hemocytometer counts for 11 Holstein cows and 5 beef cows > 2 yr, lactating, and seropositive for bovine leukemia virus. The first syringe-full (5–10 mL) was often discarded to reduce blood contamination; RBC concentrations were 5–1930/μL with a mean of 190/μL.[13]
[e] The TNCC was measured by using an unspecified hemocytometer method. Protein was assayed with QuanTtest using a human protein standard (70% albumin, 30% globulin). RBC concentrations were 0–1360/μL.[14]
A-O, atlanto-occipital; CBB, Coomassie brilliant blue; PS, Ponceau S; TCA, trichloroacetic acid

E. CSF has a very low total [protein], approximately one-hundredth to two-hundredths that of plasma, depending on the species.
F. CSF [glucose] is typically lower than plasma [glucose], increasing and decreasing in parallel with plasma changes.[15,16]

IV. Goals of CSF analysis
- A. To detect or confirm a neurologic disorder when one is suspected or is a differential for clinical findings (e.g., a recumbent dairy cow)
  1. The presence of CSF abnormalities supports neurologic disease.
  2. The absence of CSF abnormalities does not exclude neurologic disease.
  3. Meningeal and ventricular lesions are more likely to yield abnormal CSF results than deeper parenchymal lesions, but deep-seated lesions may affect CSF given the interconnectivity of CNS compartments.
  4. CSF abnormalities may be detected with structural CNS disorders – those with vascular, inflammatory, traumatic, neoplastic, or degenerative lesions – but they are not expected with disorders of a functional (e.g., epilepsy) or metabolic (e.g., hypoglycemia, hepatic or uremic encephalopathy) nature alone.
- B. To characterize neurologic disease that is clearly present
  1. The presence or absence of particular abnormalities reflects the ongoing pathologic processes and their location.
  2. Better characterization reduces the list of potential causes and directs patient management.
- C. To provide a specific diagnosis
  1. Specific diagnoses include certain infectious conditions (e.g., bacterial meningitis, cryptococcosis) and neoplastic conditions (e.g., lymphoma).
  2. Most CSF samples do not provide a specific diagnosis.
     a. There are many different neurologic disorders but relatively few possible changes to CSF as routinely assessed.
     b. Alterations from a single disorder may vary considerably with CNS location (e.g., meningeal versus deep), severity, and stage of disease.
  3. Special CSF tests may yield a diagnosis when routine testing alone does not.
- D. To determine the suitability of the CSF for further testing, e.g., verifying little RBC contamination before submitting samples to be immunoblotted for evidence of equine protozoal myeloencephalitis, as a small amount of blood contamination can yield false positive results for anti-*Sarcocystis neurona* immunoglobulin G (IgG) in the CSF[17]

V. Challenges of CSF analysis and interpretation
- A. CSF abnormalities may change with CSF turnover and changes in disease duration, location, and severity, making interpretation of a single CSF sample more difficult.
- B. CSF findings are usually not specific for particular disorders.
- C. It can be difficult to collect CSF with no or minimal blood contamination, and iatrogenic hemorrhage complicates interpretation of chemical and cytologic findings.
- D. It can be difficult or impossible to collect enough volume for all desired testing (routine and special). Small samples limit testing in smaller patients.
- E. Low TNCC and few cells in cytologic preparations limit precise and accurate nucleated cell analysis.
- F. Cell deterioration may occur before sample analysis because cells degrade rapidly in low-protein, low-lipoprotein fluids like CSF. If CSF cannot be processed and analyzed within an hour of collection, measures can be taken to help stabilize the sample for later analysis (see Sample Collection and Processing).
- G. Low protein and cell concentrations of CSF necessitate special procedures. Appropriate analytical methods and expertise are not available in many clinical settings.
- H. Rigorous, method-specific reference values for CSF are not available.
- I. Interpretive classifications and definitions for CSF abnormalities vary, thus impairing communication and comparisons regarding the CSF abnormalities reported in various neurologic disorders.

SAMPLE COLLECTION AND PROCESSING

I. Collection
   A. CSF is most often collected from the cerebellomedullary cistern through the atlanto-occipital space or from the subarachnoid space at the lumbosacral junction. Equine samples are also collected from the subarachnoid space accessed between the first and second cervical vertebrae (atlantoaxial collection).
   B. The site of collection should be recorded because there may be differences in CSF content at different locations, e.g., the [protein] of CSF was greater in lumbar samples than in samples from the cerebellomedullary cistern of healthy dogs.[5] In another study, CSF [protein], TNCC, and [RBC] differed between paired cerebellomedullary and cisternal sites in many dogs with neurologic disease, whether intracranial, cervical, or thoracolumbar in location.[18] Cell proportions and a pathologist's interpretation also varied with the site, though only single cytocentrifuge preparations made from only 100 µL of CSF were evaluated, and this might not provide adequately representative populations for cytologic assessment (authors' experience).
   C. Sampling at the site nearest or caudal to the suspected lesion is often recommended (e.g., lumbosacral collection for a focal spinal lesion), although results of routine analysis are expected to be similar for diffuse or widely multifocal conditions. The smaller the lumbosacral volume, the more it reflects only distal spinal CSF; the larger the volume, the more it reflects proximal CSF. Multiple studies support lumbar collection for detecting abnormalities associated with thoracolumbar disease in dogs.[18,19] However, for 13 of 22 dogs with intracranial or cervical disease and pleocytosis in one of these studies, pleocytosis was present at only one of the sites (usually lumbar), prompting the authors to suggest sampling both sites for suspected intracranial and cervical lesions.[18] Collection from both sites improved detection of inflammation supportive of steroid-responsive meningitis-arteritis in dogs.[20]
   D. Collection of 0.2 mL/kg is reportedly safe and allows for collection of at least 1 mL in most dogs and cats.[21] Greater volumes can be collected from the atlanto-occipital site than from the lumbosacral site, allowing for more testing.
   E. Blood contamination may occur more often with lumbosacral collection.
   F. Collection should precede contrast myelography because contrast materials (e.g., iohexol, iopamadol, metrizamide) can induce mild inflammation with increases in protein, WBC, and neutrophil concentrations; these changes can be present for 1–2 wk.[22–26]
   G. Routine CSF results were not affected by weekly repeated collections of CSF from the cerebellomedullary or atlantoaxial spaces of healthy horses.[27]

II. Sample container
   A. Sterile plain tubes are recommended.
      1. Clot tubes may be used if they do not contain a clot activator or other additive that can cause preanalytical errors; additives can shrink cells or affect chemical testing, and clot activating particulates can be phagocytized and alter cell appearances.
      2. Plastic tubes are considered safer than glass and may reduce platelet and WBC adherence and activation. They should not contain silica or other additives.[28]
   B. Tubes containing EDTA may also be used if the laboratory measures protein concentration by a method for which EDTA is not an interferent (see Routine CSF Analysis section). EDTA-anticoagulation would be useful to prevent clotting in samples with substantial iatrogenic hemorrhage or protein exudation.

III. Sample handling
   A. For locally analyzed samples, the determination of cell concentrations, generation of microscopic preparations, and separation of fluid from cells by centrifugation should occur as soon as possible, preferably within 1 h of collection.
      1. Samples may be kept at room temperature during this short time.
      2. Once separated from cells, the fluid may remain at room temperature if analyzed within a few hours, but it should be refrigerated if analyzed later, Aliquots of post-centrifugation supernatant may be frozen for chemical analysis.
      3. Results of one study of 26 canine CSF samples suggest that delaying CSF analysis for up to 8 h may have little impact on cytologic interpretations, but prompt evaluation is still preferred because of cell deterioration.[29] Cells deteriorated more slowly when the CSF [protein] was clearly increased, but [protein] is not known before analysis.
   B. For delayed microscopic analysis at an outside laboratory, after consulting with laboratory personnel in advance to learn their preferences, an aliquot of CSF may be transferred from a well-mixed sample to a similar plain tube to which is added one of the following to limit cell deterioration and lysis:
      1. One drop of autologous serum per 0.5–1 mL CSF: The effect of dilution on cell concentrations is < 10 %. Shipping in an insulated cooled container is recommended to limit cell deterioration, but evidence of cell deterioration was present after 12 h and by 24 h in refrigerated serum-treated samples of equine CSF.[30]
      2. An equal volume of hetastarch for samples that are too small (< 0.5 mL) to split into aliquots: The protein concentration can be measured but must be corrected for the dilution.[29] Shipping in an insulated cooled container is recommended to limit cell deterioration. VetStarch, a specific hydroxyethyl starch product, was not effective in preserving cells in canine CSF samples with [protein] < 50 mg/dL when assessed after 48 h of storage at 4 °C in glass tubes containing EDTA.[31] The CSF:VetStarch ratio was 2:1; preservation was as poor as with saline addition and not as good as with fetal calf serum.
      3. Ethanol (1:1 by volume) or 10 % formalin (1–2 drops per 1–2 mL CSF) may also be accepted in aliquots intended for cytologic evaluation, despite formalin's effect on cell staining. When at least 0.3 mL of canine CSF was added to 1 mL EDTA tubes containing a drop of 10 % buffered formalin and analyzed 6–20 h later after transport to a laboratory, pathologists' reports supported improved diagnostic quality compared to paired samples in EDTA tubes without formalin.[32]

ROUTINE CSF ANALYSIS

Routine CSF analysis involves only a few tests with limited abnormalities (Table 21.4). Other than [protein], all can be assessed in a practice setting, though training and experience with CSF samples are important prerequisites. Color and clarity can be readily assessed, [RBC] and TNCC can be determined if staff are trained to use a hemocytometer, and microscopic preparations can be produced by sedimentation and either mailed out for evaluation or assessed in-house if someone has adequate experience to do so. In place of determining the CSF [protein], a less accurate Pandy test or urine dipstick can be used to screen for increased [protein]. For samples with limited volumes, tests may need to be prioritized based on clinical findings and differential diagnoses. TNCC and cytologic evaluation are often the priorities, but [protein] may be a priority in some patients.

Table 21.4. General significance of major abnormalities detected during routine CSF analysis

Assessment	Finding	Significance
Color[a]	Pink to red	Sample contamination (bloody tap) Hematorrhachis (RBCs or free hemoglobin)
	Xanthochromia	Bilirubin from previous hematorrhachis, hyperbilirubinemia, high [protein] (bilirubin bound to albumin); increased carotenoids, melanin, or methemoglobin
Clarity	Turbidity	↑ [RBC], ↑ TNCC, fibrin, epidural fat
[Protein]	↑	Sample contamination (bloody tap) Inflammation or other causes of increased vascular permeability, tissue degeneration, ↑ production by lymphocytes, hematorrhachis, ↓ CSF drainage
[RBC]	↑	Sample contamination (bloody tap) Hematorrhachis (trauma, infectious and noninfectious inflammation, neoplasms, hemostatic defects)
TNCC	↑	Sample contamination (bloody tap) Inflammation (various types), neoplasms (various types)
Microscopic evaluation	Abnormal types or concentrations of cells or other structures	Sample contamination (bloody tap) Infections, noninfectious inflammation, neoplasms, hematorrhachis, epidermoid cysts (rare)

[a] Colors may be mixed when multiple abnormalities are present. Rifampin administration may impart an orange-red color with sufficient penetration into the CSF.

I. Definitions

A. The terms *large mononuclear cell* and *small mononuclear cell* are often used to refer to nongranulocytic cells, segregated by size. In CSF, these may include lymphocytes of various sizes, monocytes, macrophages, epithelial cells, and leptomeningeal cells. The authors do not use these terms because the vast majority of cells can be classified more specifically and informatively when a well-stained, quality cytocentrifuge preparation is examined (and granulocytes have one nucleus and are therefore also mononuclear).

B. *Pleocytosis*, from Greek *pleion* (more) + *kytos* (cell) + *–osis* (condition), is an increase in CSF TNCC. The term is often modified by the main cell type(s) causing the increase (e.g., lymphocytic pleocytosis, neutrophilic pleocytosis), whether caused by inflammation or neoplasia.

C. The suffixes *–rrhachia* and *–rrhachis* (from Greek *rháchis* meaning spine, ridge, backbone) are used to refer to bilirubin in the CSF (*bilirrhachia*), hemorrhage into the CSF (*hematorrhachis*), decreased or increased [glucose] in the CSF (*hypoglycorrhachia* or *hyperglycorrhachia*, respectively), or increased [protein] in the CSF (*hyperproteinorrhachia*).

D. *Xanthochromia* is descriptive for CSF with a yellow color (Greek *xanthos*, yellow, blond + *chromos*, colored), though some people also apply the term to samples that are pink or orange.

II. Physical examination
- A. Color is assessed before and after centrifugation. CSF is colorless in health. A change from pink or red to colorless upon centrifugation indicates iatrogenic or recent pathologic hemorrhage.
  1. A yellow color after centrifugation is evidence of bilirrhachia from:
     a. Degradation of hemoglobin in the CSF, which may occur within hours of local hemorrhage
     b. Systemic hyperbilirubinemia; kernicterus (bilirubin encephalopathy) may occur in neonates with increased hemolysis and marked unconjugated hyperbilirubinemia because of an undeveloped blood–brain barrier[33]
     c. Moderate to marked increases in CSF [protein] caused by leakage of plasma proteins (including albumin complexed with bilirubin), pathologic hemorrhage, or iatrogenic hemorrhage
  2. Increased plasma carotenoids or melanin from a melanoma may also impart a yellow to brown or blackish (melanin) color.
  3. A pink, orange, or red color occurs with free oxyhemoglobin from either in vivo RBC lysis after hemorrhage (may occur within 1 h) or from in vitro hemolysis, which is most likely to occur in samples not processed within 1 h. An inconsistent red color of CSF during collection (red becoming colorless or vice versa) suggests iatrogenic hemorrhage.
  4. A brown color may reflect the presence of methemoglobin forming from oxidation of oxyhemoglobin; this is more likely with bleeding into a region that cannot drain normally.
  5. Combinations of hemoglobins and bilirubin (rarely melanin) can yield color variations.
  6. Rifampin, a red-orange lipophilic antibiotic, can penetrate the CSF or enter bound to protein and impart an orange-red color. Because it is normally highly protein bound in plasma, more may be free to penetrate the CNS in hypoproteinemic patients.
  7. Spectrophotometry has been used to differentiate CSF pigments.[34]
- B. Clarity (turbidity) is assessed before centrifugation by viewing printed material through the fluid in a clear collection or microhematocrit tube.
  1. A hazy or cloudy (turbid) appearance is abnormal and usually caused by increased cells (nucleated cells or RBCs), but cell concentrations may be substantially increased without turbidity.
  2. Any suspended element like microorganisms or epidural fat may contribute. CSF should be collected prior to injection of contrast materials that may cause turbidity.
  3. Evidence was not found to substantiate the common statement that high protein concentrations may cause turbidity of CSF; protein concentrations 70–100 times greater than those in CSF do not make serum or plasma turbid.
  4. Clots may form from iatrogenic hemorrhage or a marked exudative process and contribute to turbidity.
- C. Viscosity of CSF is not consistently assessed, but CSF samples may be viscous because of capsular mucopolysaccharide of cryptococcal yeast, mucus from a metastatic carcinoma, or unintended collection of nucleus pulposus.

III. Specific gravity
- A. CSF specific gravity may be determined by refractometry using the scale calibrated for urine specific gravity, but it is of little value because:
  1. The concentrations of solutes (e.g., electrolytes, glucose, urea) in CSF change very little in pathologic states, and thus the refractometric specific gravity values change very little.

2. Even in pathologic states, CSF [protein] is so low that it causes only a minimal increase in refractive index. Note: A [protein] of 1 g/dL (which would be extreme for CSF) increases the refractometric specific gravity only about 0.004.

B. CSF specific gravity is usually in the 1.004–1.008 range, depending on the species and the calibration of the refractometer.

C. Refractometric specific gravity values may be increased if CSF is collected and analyzed after injections of contrast media; the material increases the refraction of light.

IV. Total protein concentration

A. Physiologic processes

1. Most CSF protein is of plasma origin, but small amounts are synthesized and secreted by choroid plexus epithelium.
2. Protein enters CSF at the choroid plexus and by diffusion and limited pinocytosis across capillary endothelial cells throughout the CNS. The [protein] of CSF in the ventricles is therefore expected to be at least mildly lower than that of lumbar CSF. The [protein] in canine cerebellomedullary samples is generally greater than that of lumbar samples in health and CNS disease.[5,18] The median CSF [protein] in equine atlantoaxial samples was approximately twice that of atlanto-occipital samples.[27]
3. As is true of plasma, the single most abundant CSF protein is albumin. Protein moves from brain and spinal cord to CSF and from blood to CSF by diffusion, a process that favors smaller proteins like albumin over larger ones like immunoglobulins and many other globulins.
4. Immunoglobulin G (IgG) is the major immunoglobulin in CSF, arriving from plasma.
5. CSF [protein] is transiently high in neonates. In a study of foals < 42 d of age, CSF [protein] was greatest in foals < 2 d old and decreased to adult values by 3 wk.[35]

B. Pathologic states may increase the [protein] in CSF by:

1. Increased entry of plasma proteins from blood or tissue
2. Local synthesis of immunoglobulins
3. Decreased CSF turnover rate and decreased resorption of CSF proteins

C. Analysis

1. Because CSF protein concentrations are very low, refractometry and assays for serum or plasma total protein concentrations cannot be used.
2. Special protein assays are used instead. These are sometimes called microprotein assays, but they are assays for small protein concentrations, not small proteins.
   a. Different laboratories use different assay methods, different dyes with different relative affinities for albumin and globulins, and different protein standard solutions (calibrators), so results vary, and one reference cutoff will not apply to all laboratories (Table 8.17).
   b. In general, the URL for CSF protein in dogs and cats is approximately 25–45 mg/dL. When compared directly in dogs,[36,37] lumbar taps yielded CSF protein concentrations up to about twice those of cisternal taps, but some reported limits for cisternal [protein] in dogs are as high as those for lumbar samples in other reports (Table 21.2). In most reports, the URL in horses is approximately three times that of the dog when comparing similar assays, with values as high as 120 mg/dL. Reported values in adult cattle and llamas have been intermediate, a little less than double the canine values (Tables 21.2 and 21.3), but values in 8-wk-old calves were lower and similar to those of dogs.[38] Method-specific laboratory RIs or decision limits should be used when available, but opportunities to generate dependable RIs for CSF [protein] in an appropriate number of healthy animals are limited.

3. K_3EDTA falsely increased CSF [protein] when measured by a modified biuret method on a Vitros 500 analyzer, but not when measured by a spectrophotometric Coomassie blue-binding technique (authors' unpublished data).
   a. Fresh nonanticoagulated CSF from 10 healthy and sick horses yielded 39–126 mg/dL protein, whereas paired samples in K_3EDTA yielded 91–171 mg/dL, the increases being 34–54 mg/dL and therefore of potential clinical significance.
   b. EDTA, like protein, may dissociate the cupric ion from the copper–azo dye complex in the test slides. Experimentally, the apparent [protein] increased in solutions of increasing [NaCl] but constant [K_3EDTA] (3.7 mM), suggesting that the false increases in CSF [protein] were facilitated by ions in CSF.
4. VetStarch, when tested as a cell preservative in canine CSF, markedly increased CSF [protein] when measured with the Vitros modified biuret method (copper–azo dye complex) but not with a Coomassie blue-binding method.[31]
5. Urine dipsticks have been used for protein analysis of CSF. Reactions of at least 2+ support increased protein in dogs, but mild increases may or may not be present with a 1+ reaction.[39,40] Values as great as 2+ occurred in healthy horses.[12] Quantitative assays are recommended.

D. Increased CSF [protein] is a nonspecific finding but evidence of neurologic disease if not caused by a traumatic tap (Table 21.5).

E. Documenting decreased CSF [protein] may be difficult considering the analytical properties of microprotein assays and the reported variations in expected concentrations in domestic mammals. However, CSF [protein] may be diminished by increased intracranial hypertension causing increased resorption of CSF, including proteins. Also, lumbar CSF [protein] may decrease with increased removal or leakage of CSF.

V. RBC concentration

A. As RBCs are not expected in the CSF of healthy animals, few to many reflect either iatrogenic or pathologic hemorrhage. These may be distinguishable by the pattern of bleeding during collection, or by the cytologic detection of hemosiderin or phagocytized RBCs with pathologic hemorrhage. Lack of platelet detection is not strong support for

Table 21.5. Disorders and conditions that cause increased CSF [protein]

Increased vascular permeability
Infectious or noninfectious inflammation of the meninges, with or without inflammation of deeper tissues*
Hypothyroidism[41]
Diabetes mellitus
Necrosis or degeneration of CNS tissue
Increased local production of immunoglobulins
Inflammatory disease
Plasma cell or B lymphocyte neoplasm
Decreased protein resorption due to obstruction of CSF flow by masses or high venous pressure
Pathologic hemorrhage into the CSF
Primary or metastatic neoplasms (by any or all of the mechanisms listed above)
Traumatic tap with iatrogenic hemorrhage or plasma contamination*

* A relatively common disorder, condition, or mechanism

pathologic hemorrhage, because platelets may have adhered to the collection vial or lysed during sample handling. The presence of platelets would suggest collection-induced hemorrhage or very recent to ongoing pathologic hemorrhage.

B. Besides being a potential indicator of hemorrhage, [RBC] may be relevant to interpretation of CSF [protein], TNCC, and cell differentials, as the presence of blood can significantly alter these values.

C. Measurement is usually done by a hemocytometer method, but automated counting may be done using protocols developed for high-end hematology analyzers (not point-of-care analyzers).

1. Hemocytometer (see upcoming Section VI.D.1 and Figure 21.2)
2. Hematology analyzer: CSF methods have been developed for several analyzers, but there has been little rigorous testing for veterinary samples. Compared to a hemocytometer, the ADVIA requires more CSF volume and has similar imprecision at low [RBC]. The authors of one study considered the two methods to have an acceptable level of agreement for canine samples, but there were a few substantial unexplained discrepancies (e.g., ADVIA values at least twice hemocytometer values).[42] Meaningful comparisons are limited by the imprecision of low-concentration measurements for both methods. Similarly, unless modified since this writing, the Sysmex XN-V hematology analyzer with body fluid module reports [RBC] to the nearest 1000/μL, thus preventing accurate measurement of lower concentrations.[43]
3. Cytologic estimates of RBC-to-nucleated-cell ratios can be used as very crude checks on quantitative results (e.g., seeing many more RBCs than nucleated cells would question any TNCC and [RBC] data to the contrary).

VI. Total nucleated cell concentration (TNCC)

A. Because very few nucleated cells in CSF are not WBCs, the TNCC is often a [WBC] and is often referred to as a [WBC] or WBC count.

B. The difference between counts and concentrations is particularly important in the context of CSF samples, though many people loosely refer to concentrations as counts. At least in human medicine, nucleated cells are sometimes reported as counts. i.e., the number of cells counted in a hemocytometer chamber, rather than as concentrations, i.e., the number of cells per volume calculated from counts. Using a Fuchs–Rosenthal hemocytometer and a sample with a total of 16 nucleated cells in the 3.2 μL grid volume, the count that some sources report would be 16, whereas the concentration would be 16/3.2 μL = 5/μL.

C. Recognizing an increase in TNCC is very helpful in detecting neurologic disease. Moderate to marked increases in TNCC are typically clear, but definitive identification of mild increases is hampered by:

1. Uncertainties in expected values in health (e.g., reported URLs range from 2/μL to 7/μL for dogs)
2. Imprecision and inaccuracies of measuring low cell concentrations
3. Uncertain contributions of nucleated cells due to blood contamination

D. TNCC is usually measured by hemocytometer, but several hematology analyzers with CSF protocols and controls have been evaluated for human or veterinary samples.

1. Hemocytometer method (Figure 21.2): TNCC and [RBC] are calculated from nucleated cell counts in specified hemocytometer grid areas. Incubation and mixing of CSF in a plain capillary tube previously internally coated with negligible volumes of new methylene blue can be used to stain nuclei and better differentiate nucleated cells (especially small lymphocytes) from RBCs. Fuchs–Rosenthal hemocytometers have been recommended for CSF samples because the depth of the counting chamber is

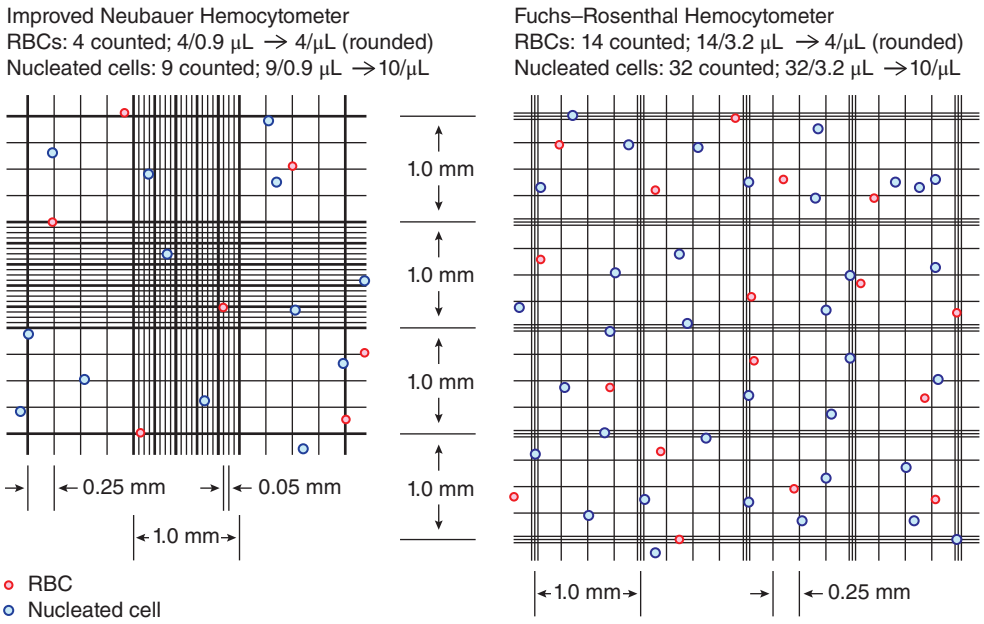


Figure 21.2. Neubauer and Fuchs–Rosenthal hemocytometer grids for measuring CSF cell concentrations.

- For both types of hemocytometer, a clean cover glass is placed over the recessed, etched counting grids shown here. The space under the cover glass and over the grids on each side of the hemocytometer are filled with 10–20 µL of well-mixed, undiluted CSF (± stain) using a pipette and capillary action.
- The cells are typically allowed to settle for a few minutes before microscopic evaluation with the condenser somewhat lowered or the iris diaphragm partially closed to promote cell visibility. After finding and focusing on the grid using the 10× objective, 20–45× objectives are typically used for counting the nucleated cells and the RBCs (separately), typically in all 9 (Neubauer) or 16 (Fuchs–Rosenthal) large squares; the greater-magnification objectives are needed to reliably differentiate RBCs from nucleated cells such as lymphocytes.
- Although it is recommended that at least 100 cells be counted per grid, CSF often contains fewer cells than this. If cell concentrations are increased, cells can be counted in a subset of the large squares, but they should be selected symmetrically and the number of counted cells must be divided by 0.1 µL times the number of large squares counted. Dilutions and subsequent calculations to correct for the dilution are recommended with cell concentrations > ~2500/µL (i.e., 25 cells per large square). Cells that touch the squares' borders are counted on only two of the sides, typically the top and left, so that they are not counted in more than one square. After counting cells in one grid area, cells are counted in the other grid area and the results are averaged if there is good agreement between sides.

In the example shown here with schematic cells (not shown to scale), one sample has been loaded into both a Neubauer hemocytometer (*left*) and a Fuchs–Rosenthal hemocytometer (*right*).

- In the Neubauer grid, each large square represents 0.1 µL (1 mm × 1 mm × 0.1 mm depth). With nine large squares, the total chamber volume is 0.9 µL.
- In the Fuchs–Rosenthal grid, each large square represents 0.2 µL (1 mm × 1 mm × 0.2 mm depth). With 16 large squares, the total chamber volume is 3.2 µL.

With the Fuchs–Rosenthal hemocytometer, more nucleated cells and RBCs are counted in each grid, while still using very little CSF.

twice that of the Neubauer hemocytometer (0.2 mm versus 0.1 mm), and more area is assessed (16 mm^2 versus 9 mm^2 per chamber). Therefore, when using both grid chambers, 6.4 µL of CSF are evaluated with the Fuchs–Rosenthal hemocytometer versus just 1.8 µL for the Neubauer hemocytometer. Other counting options are

available, such as disposable Fast-Read 102 plastic counting chambers which have a 1 μL sample volume over the grid area.

2. Hematology analyzer
   a. When comparing TNCCs measured using a Neubauer hemocytometer with those measured using a Sysmex XN-V hematology analyzer with body fluid module (veterinary software 3.04), classification as pleocytosis present (TNCC > 5/μL) or absent agreed in 141 of 150 samples, seven samples (4.6%) having mild increases with the Sysmex (7–10/μL) but not with the hemocytometer (0–4/μL) and two samples (1.3%) having TNCCs of 3/μL with the Sysmex but 6–20 cells/μL (pleocytosis) based on the hemocytometer.[43]
   b. Unless the software has been modified, the ProCyte Dx reports CSF TNCC in increments of 10/μL, with 10/μL being the lowest reported concentration, making the analyzer unable to differentiate mild increases from no increases.[44] Accuracy data for the greater concentrations is difficult to interpret given that there was up to a 24 h delay before assessment with the comparison method.
   c. TNCCs generated by the ADVIA 2120 for canine CSF samples had good correlation with paired Fuchs–Rosenthal hemocytometer values but had a positive bias at low concentrations (i.e., < 10–15/μL) that led to differences in which samples were classified as having pleocytosis.[42] The methods had similar imprecision, but the analyzer required more sample volume (300 μL). Studies comparing human CSF TNCCs between Fuchs–Rosenthal and Sysmex XE-5000 (150 μL sample) methods yielded similar degrees of imprecision and a positive bias of approximately 2–3/μL for the XE-5000.[45]
3. Microscopic estimates of RBC-to-nucleated-cell ratios on cytologic preparations can be used as very crude checks on quantitative results (e.g., if lymphocytes outnumber RBCs on cytocentrifuge preparations but a TNCC was determined by hemocytometer to be 0/μL, some lymphocytes may have been counted as RBCs).
4. Because a much larger volume is used for cytocentrifuge preparations (e.g., 200 μL) than for hemocytometer counts (≤ 6.4 μL), cells may be found in a cytocentrifuge preparation when none are found in the hemocytometer.

E. Pleocytosis can occur with many inflammatory or neoplastic disorders (Table 21.6). When pleocytosis is present (or absent), microscopic evaluation of cytologic preparations may reduce the list of differentials or yield a specific diagnosis.

VII. Effect of blood contamination on quantitative analyses

A. Because at least mild iatrogenic hemorrhage is common with CSF collection, it would be helpful to know the influence of blood contamination on TNCC and [protein]. Theoretically, TNCC or protein correction formulas could be applied to samples

Table 21.6. Disorders and conditions that cause pleocytosis[a]

Infectious inflammation:* bacteria, fungi, viruses, parasites, or protozoa
Noninfectious inflammation:* necrosis/degeneration, trauma, foreign material, vasculitis, toxicity, neoplasms, metabolic disease, immune-mediated disease
Hemorrhage (pathologic or from collection)
Neoplasms (increased neoplastic cells), primary, metastatic, or multicentric

* A relatively common disorder, condition, or mechanism

[a] Cytologic findings are used in conjunction with clinical information to generate a more focused list of differential diagnoses for samples with pleocytosis.

containing increased RBCs. However, calculations do not reliably predict effects of blood contamination on TNCC and [protein],[46] likely, in part, because technique-related vascular contamination of CSF may not yield proportional additions of protein, WBCs, and RBCs; more plasma than cells may leak, or cells may be marginated and enter at greater concentration than in venous blood. Also, the degrees of pathologic versus iatrogenic hemorrhage may be unknown, and there may be differences in contaminating blood composition from that of venous blood sampled for a CBC or reference samples used to create formulas. So, iatrogenic hemorrhage should be minimized when collecting CSF.

B. Calculations can provide only crude guidance for expected effects of contamination with certain volumes of blood having particular cell and protein characteristics. The following examples use blood values expected in health, which may not be applicable to patients.
   1. A 20 μL drop of "healthy blood" within a total CSF+blood volume of 1 mL would increase the TNCC by 140/μL, the [RBC] by 140,000/μL, and the [protein] by 77 mg/dL (Eqs. 21.1 and 21.2). One-fifth of this, just 4 μL of blood, would increase the TNCC by 28/μL, the [RBC] by 28,000/μL, and the [protein] by 15 mg/dL, all of which could be significant.

If the $[protein]_{plasma}$ = 7 g/dL, blood Hct = 45%, and there is a 1 mL CSF sample containing 0.02 mL (20 μL) of blood, then:

$[protein]_{blood}$ = $[protein]_{plasma}$ × (1 – Hct in decimal form)

= 7 g/dL × (1 – 0.45) = 3.85 g/dL = 3850 mg/dL = 38.5 mg/mL

Protein added by blood = $[protein]_{blood}$ × added blood volume (**21.1**)

= 38.5 mg/mL × 0.02 mL = 0.77 mg

If the $[protein]_{CSF}$ prior to added blood is 20 mg/dL (0.2 mg/mL) and the 1 mL sample of CSF contains 0.77 mg of protein from blood, then the contaminated CSF sample has a [protein] of 0.97 mg/mL = 97 mg/dL.

If a blood [WBC] is 7000/μL, the blood [RBC] is 7,000,000/μL, and there is a 1 mL CSF sample containing 0.02 mL (20 μL) of blood, then:

RBCs added by blood = $[RBC]_{blood}$ × added blood volume

= 7,000,000/μL × 20 μL = 140,000,000 RBCs

If the $[RBC]_{CSF}$ prior to added blood is 0/μL (=0/mL) and the 1 mL sample of CSF contains 140,000,000 RBCs from blood, then the contaminated CSF sample has a [RBC] of 140,000,000/mL = 140,000/μL.

(**21.2**)

WBCs added by blood = $[WBC]_{blood}$ × added blood volume

= 7000/μL × 20 μL = 140,000 WBCs

If $[WBC]_{CSF}$ prior to added blood is 0/μL (= 0/mL) and the 1 mL sample of CSF contains 140,000 WBCs from blood, then the contaminated CSF sample has a [WBC] of 140,000/mL = 140/μL.

2. Given a ratio of ~1000:1 for canine blood [RBC]:[WBC] in health, the TNCC in a CSF sample with iatrogenic hemorrhage and a CSF [RBC] of 10,000/μL could be increased 10/μL because of iatrogenic hemorrhage, and the [protein] could be increased 6 mg/dL. Only a 1/μL increment in TNCC would result from iatrogenic hemorrhage yielding a 1000/μL [RBC], and such a small increment would be undetectable given the imprecision of TNCC measurements. The increment would theoretically be 5/μL if the patient had a blood [WBC] of 50,000/μL, and the effect on [protein] would be negligible.

VIII. Microscopic examination

A. Microscopic evaluation of stained air-dried cytopreparations is an important component of CSF analysis, and abnormal findings may be present whether or not cell concentrations are increased.

B. Because of low cell concentrations, even in disease states, appropriate cytopreparations typically rely on either gravitational sedimentation[6,47] or, more often, cytocentrifugation to concentrate the cells. A small amount of protein may be added to the cytocentrifuge sample to stabilize the cells and improve their preservation (e.g., 1 drop of 22 % bovine serum albumin added to 200 μL CSF). Because cell recovery is poor and inconsistent,[48] duplicate preparations are preferable, particularly if using as little as 200 μL per slide. Volumes of 300–500 μL may be preferable, if available, for single or duplicate preparations.

C. Smears of resuspended sediment after centrifugation in a standard centrifuge often yield a poor harvest of cells that are distorted or damaged. Concentrated preparations may also be made without purchase of a cytocentrifuge by using a modified salad-spinner method.[49]

D. After air-drying, preparations are generally stained with a Romanowsky stain; special stains may be applied as indicated if sample volume allows.

E. A major component of the cytologic examination is generating a nucleated cell differential count. Although 100 or 300 cell differentials are common goals, fewer than 100 nucleated cells are often present, even when cells from two preparations are totaled. In such cases, reporting only the percentages of each cell type may be misleading without revealing the low number of cells they are based on, as the imprecision of differential percentages of low-percentage cells is very high.[48]

F. Nucleated cell types in health (Figure 21.3)

1. Nucleated cells expected in health are mostly small lymphocytes and monocytoid cells that are variously referred to as monocytes, macrophages, monocytes/macrophages, reticulomonocytes, or mononuclear cells. So-called mononuclear cells may be subclassified as large mononuclear cells and small mononuclear cells, with the small mononuclear cell category often including lymphocytes in addition to small monocytes/macrophages. This is because differences between some lymphocytes and some monocytes/macrophages can be relatively subtle, particularly in cytocentrifuge preparations. Unfortunately, use of "mononuclear" varies and is not always defined (note: all these cells generally have one nucleus and are therefore mononuclear; macrophages are most likely to have multiple nuclei). Other cell types (e.g., ependymal or choroid plexus cells) may be included under the "large mononuclear cell" umbrella, but they have a distinct appearance.
2. Low numbers of neutrophils and rare eosinophils may be present, especially with blood contamination; with low total numbers of nucleated cells to evaluate, few neutrophils can generate substantial neutrophil percentages that may be misinterpreted as reflective of disease. In 15 healthy dogs with no or trace RBCs

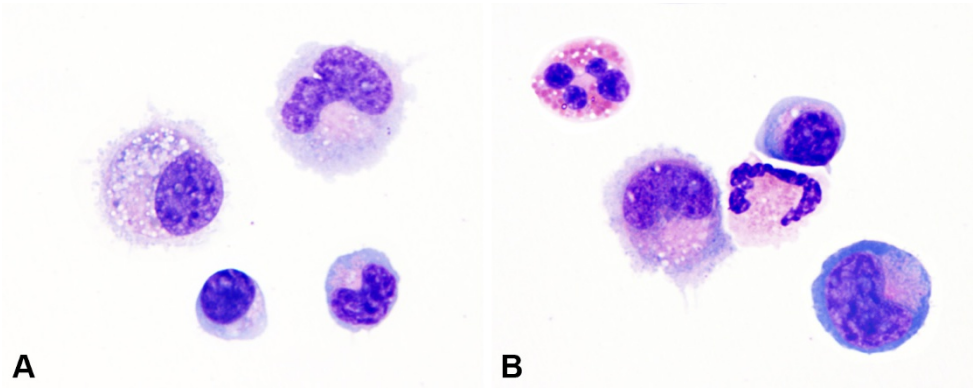


Figure 21.3. A. Cells commonly seen in CSF without disease: a monocytoid cell (*top right*), small lymphocytes (*bottom*), and a macrophage (*left*). B. An eosinophil (*top left*) or reactive lymphocyte (*bottom right*) can be present in health, but rarely. Few neutrophils can be present in health, particularly with hemorrhage during collection; one is shown here between a macrophage and a lymphocyte. Cytocentrifuge preparations, dog, 100× objective, Wright stain. Note: When cells spread flat in cytocentrifuge preparations of CSF, small lymphocyte cytoplasm can appear more abundant and neutrophil granules can stain redder than in many sample types.

(none in 14 of them), differential counts of an unspecified number of nucleated cells yielded 0–42 % neutrophils with a mean ± SE of 4.8 ± 2.8 %.[9]

3. Other cells that may be present in the CSF are low numbers of cells that line the subarachnoid space and are in contact with CSF. These are ependymal cells (ependymocytes, which are modified glial cells of neuroectodermal origin), choroid plexus cells (modified ependymal cells), and leptomeningeal cells (from mesenchyme). These cells may be individualized or in small clusters because of desmosomal connections. Leptomeningeal cells or macrophages may contain melanin in some species, especially cattle and sheep.

G. Nucleated cell types in disease; examples are provided in veterinary cytology atlases (Figure 21.4)[50–53]

1. In CNS disease, most cell types responsible for pleocytoses are those that can be seen in health: small lymphocytes, monocytoid cells, neutrophils, and eosinophils (rarely seen in health).
2. Most other cells are nonneoplastic variations of expected cell types: reactive lymphocytes, plasma cells, vacuolated macrophages.
   a. Reactive lymphocytes or plasma cells support inflammatory disease, although they rarely have been reported in clinically healthy individuals.[11]
   b. Cells can become vacuolated in vitro, so vacuolated macrophages do not necessarily indicate in vivo change.
3. Neoplastic cells are uncommonly found, even when CNS neoplasms are present, but those that may be found include lymphocytes, plasma cells, histiocytes, benign or malignant choroid plexus cells, metastatic carcinoma cells, or rarely neoplastic resident cells such as oligodendroglial cells. Immunophenotyping by flow cytometry or immunocytochemistry on concentrated preparations may be useful for identifying or characterizing certain neoplasms (e.g., lymphoid, histiocytic), though sample volume and cell concentrations may be limiting. Another option is to make cell blocks for immunohistochemistry. CSF also may be used to assess lymphoid populations for clonality by polymerase chain reactions for antigen receptor rearrangements (PARR).

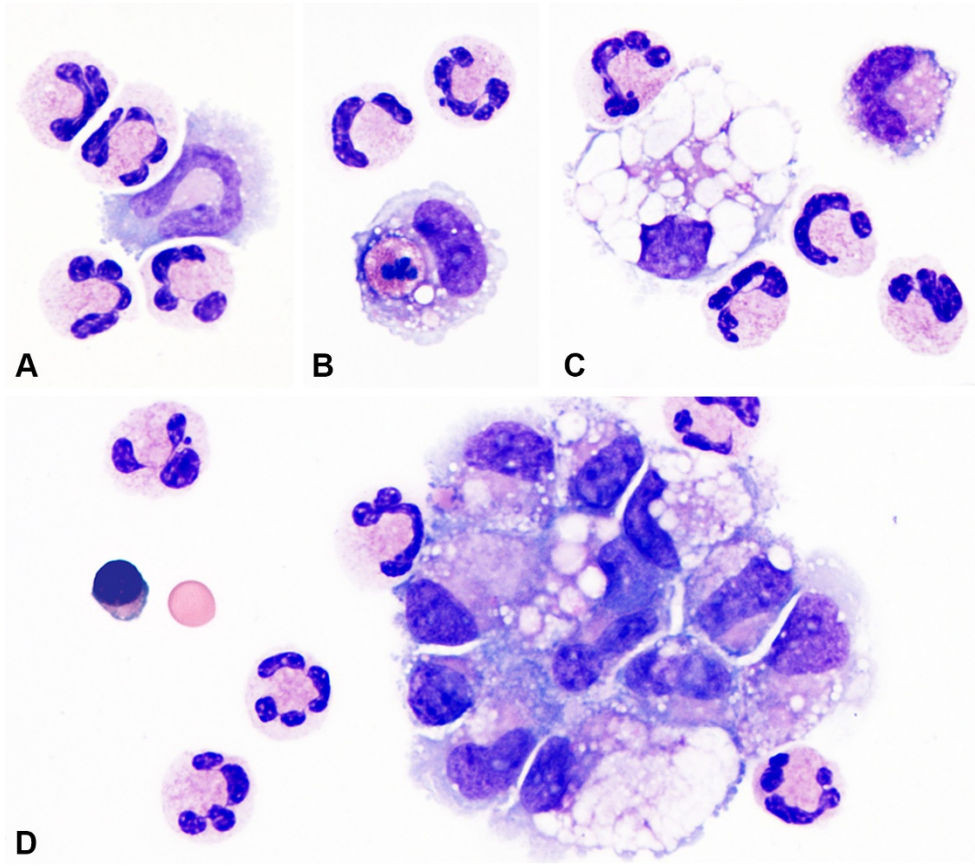


Figure 21.4. CSF with a mixed inflammatory pleocytosis including an increased [neutrophil] (A–D), a monocytoid cell (A), a macrophage containing a partially degraded neutrophil (B), a large vacuolated macrophage (C), and a cluster of macrophages (D) near a single RBC and a small lymphocyte from a dog with idiopathic granulomatous meningoencephalitis, 100× objective, Wright stain.

4. Squamous epithelial cells or squames may be seen because of skin contamination (from patient or clinician) or, rarely, sampling of an epidermoid cyst or a metastatic squamous cell carcinoma.
5. Hematopoietic cells may be present because of accidental sampling of bone marrow or collection of cells exfoliating from sites of extramedullary hematopoiesis within the CNS.
6. Other contaminants may include chondrocytes and neural tissue cells.

H. Other microscopic findings, many of which are illustrated in veterinary cytology atlases[50–53]
1. Macrophages may contain RBCs or hemosiderin, indicative of recent or previous hemorrhage.
2. Bacterial cocci or rods may be present, free or within neutrophils. Morulae may be in monocytes/macrophages, neutrophils, or eosinophils (morulae have been reported in each of these cell types in the CSF of dogs with evidence of *Ehrlichia canis* infections, despite the expectation of finding *E. canis* morulae in monocytes or lymphocytes).[54,55]

3. Fungal organisms may be present free or within phagocytes. *Cryptococcus* is most common, but other fungi that have been seen include *Blastomyces* and *Histoplasma*.
4. Other infectious agents that have been detected rarely in CSF include tachyzoites of *Neospora*, *Toxoplasma*, or *Sarcocystis*, viral inclusions of canine distemper virus, nematode larvae (*Angiostrongylus* spp. in dogs, *Halicephalobus* in horses), nematode ova (*Spirocerca* in a dog), amoebae (*Naegleria* in ruminants), trypomastigotes (*Trypanosoma* spp. in horses and cattle), and possibly unidentified microsporidia in a dog.
5. Round to amorphous globules of pink myelin-like material are relatively commonly seen extracellularly and are rarely seen within macrophages. Their extracellular presence was associated with lumbar taps, small patient size, and intervertebral disk disease, but it did not appear to be associated with progressive neurologic disease or poor outcome.[56] The significance of this material within macrophages requires further study.

I. Interpretation of cytologic findings
1. Like most complete blood counts (CBCs), most CSF analyses do not provide a specific diagnosis, but they help detect abnormalities. Detection of CSF abnormalities can help confirm neurologic disease, and the type of abnormality often provides diagnostic direction or support of a clinical suspicion.
2. Lack of abnormal findings does not exclude CNS disease, especially deep lesions unassociated with the leptomeninges.
3. Abnormal CSF without a specific diagnosis (e.g., neoplasm, infection, epidermoid cyst) is commonly classified by the type of pleocytosis (e.g., neutrophilic pleocytosis, eosinophilic pleocytosis, mixed-cell pleocytosis); such samples may alternatively be labeled as neutrophilic, eosinophilic, or mixed-cell inflammation.
4. Various inflammatory patterns are not specific for particular types of disorders. For example, neutrophilic inflammation occurs with bacterial, fungal, protozoal, viral, traumatic, degenerative, neoplastic, and immune-mediated or steroid responsive conditions.

COMMENTS ABOUT SELECTED CSF PATTERNS

When the TNCC is increased and specific diagnoses are not apparent cytologically, the latter being the usual case, CSF abnormalities are commonly said to be classified by the predominant cell type. However, there is no universal consensus for the meaning of "predominant" in classifying CSF samples. In general, "predominant" means main, most common, or most important, so it may be used to reflect either numbers or significance. One numeric definition of "predominant" is $\geq$ 70 % of the nucleated cells,[57,58] but pathologists may classify CSF based on other definitions such as > 50 % of the nucleated cells (i.e., a simple majority) or as the cell type that is clearly most abundant, whatever its percentage. The latter will be used in this chapter.

Although percentage and significance are often aligned, the most numerous cells being the most significant, minority populations may be considered most diagnostically significant in some cases (e.g., eosinophils, neutrophils), especially at relatively low TNCC when the influx of inflammatory cells is mild. Therefore, classification labels are often applied somewhat subjectively at the discretion of individual pathologists.

Disorders listed in association with each of the following patterns were derived from published reviews and many original articles, only a few of which are referenced here.[13,51–53,57,59–61] These lists demonstrate the nonspecificity of CSF patterns and provide differentials and therefore direction to facilitate searching for more specific information on each condition (Table 21.7). Such condition-related information is outside the scope of this chapter.

Table 21.7. CSF inflammatory patterns occurring with selected causes of CNS disorders[a]

Type of disorder	Neutrophilic	Eosinophilic	Lymphocytic	Mono/Macro	Mixed[b]
Infectious					
Bacterial[c]	✓	✓ (sporadic)	✓[e]	✓[e]	✓[f]
Viral	✓ (with FIP)	✓ (sporadic)	✓	✓	✓
Fungal, protozoal	✓	✓	✓ (minority)	✓	✓
Verminous		✓	✓ (minority)	✓	✓
Idiopathic noninfectious inflammatory					
Steroid-responsive meningitis-arteritis	✓			✓	✓
Granulomatous meningoencephalitis	✓ (minority)	✓ (uncommon)	✓	✓	✓
Necrotizing meningoencephalitis	✓ (minority)		✓	✓	✓
Eosinophilic meningoencephalitis		✓			
Degenerative, traumatic, vascular[d]	✓	✓ (sporadic)[g]	✓[h]	✓	✓
Neoplastic	✓	✓ (sporadic)	✓	?	✓
Idiopathic epilepsy					✓ (mild)

[a] Based on the predominant WBC, but reported eosinophilic inflammation might have been mixed inflammation with prominent eosinophils
[b] Any combinations of at least two substantially contributing WBC types, including disorders for which "mononuclear pleocytosis" was reported without differentiating lymphocytes and monocytes/macrophages; often seen with chronicity or resolution of inflammation with other patterns
[c] Includes bacterial meningitis, meningoencephalitis, meningomyelitis, discospondylitis, and abscesses adjacent to the CNS
[d] Includes intervertebral disc herniation, necrosis/myelomalacia, cerebrovascular disease with infarction, fibrocartilaginous embolic myelopathy, trauma, and hemorrhage
[e] Reported with listeriosis (cattle) or resolving bacterial infections
[f] Reported with listeriosis (cattle), ehrlichiosis (dogs), or resolving bacterial infections
[g] Reported with intervertebral disc herniation
[h] Reported with intervertebral disc herniation (particularly after about a week), ischemic cardiovascular disease, and polioencephalomalacia in cattle
Checkmarks are in bold font (✓) for patterns that are more common for the listed disorders.
FIP, feline infectious peritonitis; Mono/Macro, Monocytic/Macrophagic

I. Neutrophilic inflammation
   A. Neutrophilic inflammation is present when there is a pleocytosis with a predominance of neutrophils, and it may be present when there is an increased percentage of neutrophils without an increased TNCC. In the latter case, the greater the neutrophil percentage and less the blood contamination, the more likely there is inflammation, but the TNCC and cytologic preparation cellularity also impact interpretation (e.g., neutrophilic inflammation unclear if both are very low but clearer with higher TNCC). Also, neutrophils may contribute substantially to mixed-cell inflammation without being predominant.
   B. Neutrophilic inflammation may be caused by many CNS or systemic disorders, most of which cannot be differentiated by routine CSF analysis alone. However, in a minority of cases, detection of bacteria, fungi, or protozoa may provide a specific diagnosis. In addition, marked neutrophilic inflammation, as opposed to mild or mixed inflammation that includes neutrophils, may suggest different disorders (e.g., bacterial infection, steroid-responsive meningitis-arteritis).
   C. Conditions that have been reported in association with neutrophilic inflammation, often without a cause being apparent microscopically
      1. Infectious: bacterial meningitis or meningoencephalitis with or without empyema (including *Ehrlichia canis* infections), discospondylitis, abscesses communicating with the CSF, fungal infections, toxoplasmosis, amoebiasis, and feline infectious peritonitis virus infection
      2. Noninfectious inflammatory: steroid-responsive meningitis-arteritis (other names include canine meningeal polyarteritis, beagle pain syndrome, sterile meningitis of boxers, necrotizing meningeal arteritis, canine juvenile polyarteritis syndrome, immune-mediated meningitis) and a minority of idiopathic necrotizing meningoencephalitis (dogs) and idiopathic granulomatous meningoencephalitis (dogs) cases
      3. Degenerative, traumatic, vascular: intervertebral disc herniation, necrosis/myelomalacia, cerebrovascular disease with infarction, fibrocartilaginous embolic myelopathy, trauma, hemorrhage
      4. Neoplasms: some meningiomas
      5. Other: developmental disorders and postmyelographic aseptic meningitis
   D. Disorders that sometimes yield neutrophilic inflammation may also produce other patterns, sometimes related to the temporal stage of the condition. For example, CSF findings in bacterial meningitis may transition from neutrophilic inflammation to mixed-cell or monocytic/macrophagic inflammation with chronicity or treatment.

II. Eosinophilic inflammation
   A. Eosinophilic inflammation is present when there is a pleocytosis with a predominance of eosinophils, and it may be present when there is an increased percentage of eosinophils without an increased TNCC. In the latter case, the greater the eosinophil percentage and less the blood contamination, the more likely there is eosinophilic inflammation, but the TNCC and cytologic preparation cellularity also impact interpretation as for neutrophils. Eosinophils may also contribute substantially to mixed-cell inflammation and further diagnostic plans without being predominant.
   B. Eosinophilic inflammation with eosinophil predominance or mixed inflammation with a prominent eosinophilic component suggest a parasitic or nonparasitic hypersensitivity component to the disorder. However, it is nonspecific and may be seen with a variety of conditions.
      1. Infectious: verminous meningoencephalitis/meningomyelitis (*Baylisascaris*, *Angiostrongylus, Cuterebra*, *Dirofilaria*, *Parelaphostrongylus*, *Hypoderma*, *Spirocerca,*

other), protozoal infections (*Toxoplasma*, *Neospora*), fungal infections (*Cryptococcus*), algal infections (*Prototheca*), and, sporadically, bacterial infections and viral infections (canine distemper, rabies)

2. Noninfectious inflammatory: idiopathic eosinophilic meningitis/meningoencephalitis/meningomyelitis (dogs), idiopathic granulomatous meningoencephalitis (dogs, uncommon finding)
3. Other: sporadically with intervertebral disc herniation or neoplasms (e.g., lymphoma, primary CNS or disseminated histiocytic sarcoma, meningioma, oligodendroglioma), with or without detected atypical cell populations; potentially, based on the human model, in response to the placement of CSF shunts

III. Lymphocytic inflammation

A. Lymphocytic inflammation is present when there is a pleocytosis with a predominance of lymphocytes, and it may also contribute substantially to mixed-cell inflammation without being the predominant reaction. As small lymphocytes can predominate in health, recognition of lymphocytic inflammation requires an increased TNCC, the presence of reactive lymphoid cells, or a predominance of nonneoplastic granular lymphocytes.

B. Because lymphocytes are often lumped into a "small mononuclear" cell category, conditions with lymphocytic inflammation may be labeled as mononuclear inflammation or mononuclear pleocytosis. However, these terms do not clearly differentiate predominantly lymphocytic reactions from predominantly monocytic/macrophagic reactions. Also, the term *lymphocytic pleocytosis* does not differentiate lymphocytic inflammation from a lymphocytic neoplasm.

C. Lymphocytic inflammation may occur with infectious or noninfectious conditions, being common in viral and immune-mediated conditions. Unless organisms or neoplastic cells are detected, these conditions cannot be differentiated without other laboratory or clinical information.

1. Infectious: some bacterial infections (following antimicrobial therapy, listeriosis in cattle), viral infections (subacute or chronic canine distemper, rabies, and infections with feline immunodeficiency virus, feline infectious peritonitis virus, or West Nile virus), some fungal infections, protozoal infections (toxoplasmosis), amoebiasis, and chronic nematode infections (e.g., with *Parelaphostrongylus*, though often eosinophilic)
2. Noninfectious inflammatory: idiopathic necrotizing meningoencephalitis and idiopathic granulomatous meningoencephalitis (dogs)
3. Degenerative, traumatic, vascular, other: ischemic myelopathy, intervertebral disc herniation in some dogs (especially after several days), equine cauda equina neuritis, polioencephalomalacia in some cattle, and neoplasms

IV. Monocytic/macrophagic inflammation

A. Because the terms used for cells in the CSF vary, so does the label for an increase in monocytoid/macrophagic/large mononuclear cells. Some people use the term mononuclear inflammation or mononuclear pleocytosis. However, these terms do not clearly differentiate predominantly lymphocytic (small mononuclear) reactions from predominantly monocytic/macrophagic reactions. Others have used the term histiocytic pleocytosis. Use of varied terms makes review of the literature difficult with respect to determining the relative numbers of different cell types in various conditions.

B. Although there are phenotypic differences between monocytes and macrophages, their appearances overlap (some monocytes transition to macrophages). Cells typical of each cell type are present in many cytologic samples of inflammatory lesions within the CSF and

elsewhere, from abscesses to bronchoalveolar lavages, and they are commonly lumped together under the "macrophagic" label. However, that has not been the routine for CSF samples.

C. Monocytic/macrophagic inflammation is present when there is a pleocytosis with a predominance of monocytes/macrophages, and it may also contribute substantially to mixed cell inflammation without being the predominant reaction. Because these cells can predominate in health, the TNCC must be increased or the cells must have certain morphologic changes to indicate the presence of inflammation. Such changes include phagocytized cells or other material, multinucleation, and prominent cytoplasmic vacuoles in promptly processed samples.

D. Conditions associated with monocytic/macrophagic inflammation include most of those that can occur with lymphocytic inflammation, many that can cause neutrophilic inflammation (from trauma to rickettsial infections to other resolving infections), and many with a significant eosinophilic inflammatory component. In cattle, reported disorders include salt poisoning, listeriosis, and abscesses within or adjacent to the brain or spinal cord.

E. Monocytic/macrophagic inflammation is commonly present with nonbacterial infections (fungal, protozoal, viral, nematodal), *Listeria* infections in cattle, and idiopathic granulomatous meningoencephalitis in dogs.

V. Mixed-cell inflammation

A. When no inflammatory cell type clearly predominates in number or significance, CSF may be categorized as having mixed-cell inflammation or mixed-cell pleocytosis.

B. The terms "mixed mononuclear pleocytosis" or "mononuclear pleocytosis" have been specifically applied to CSF with a combined predominance of lymphocytes and monocytes/macrophages, a subset of a mixed-cell inflammatory process as defined here. Some have defined "mixed mononuclear" samples as having no single predominant cell type or combined lymphocytes plus monocytes/macrophages > 70 % of nucleated cells.

C. When the TNCC is not clearly increased or is only mildly increased because of increases in neutrophils or eosinophils, the pattern may look mixed when in fact the lymphocytes and monocytes/macrophages may not be increased.

D. Mixed-cell inflammation may occur at particular stages of many of the conditions noted above, sometimes with chronicity or resolution. Examples of disorders in which mixed-cell inflammation has been repeatedly reported include all the nonbacterial types of infections, *Ehrlichia canis* infection, listeriosis (cattle), idiopathic necrotizing and granulomatous meningoencephalitides (dogs), steroid-responsive meningitis-arteritis (dogs, but neutrophilic when acute), feline infectious peritonitis virus infection, canine distemper, eastern equine encephalitis virus infection in a puppy, rabies, intervertebral disc herniation, ischemic myelopathies, neoplasms, and polioencephalomalacia in some cattle. Reports of CSF findings in dogs with idiopathic epilepsy indicate that a small minority have very mild pleocytosis of nongranulocytic cells (reported as "mononuclear" without differentiating specific cell types). In cattle, "mononuclear pleocytosis" without further WBC differentiation has been reported with bovine herpesvirus 5 infections.

VI. Hemorrhage

A. RBCs are not expected in CSF from healthy individuals, but they may be present in CSF samples because of iatrogenic hemorrhage during collection. This is more likely in smaller animals and with lumbar taps.

B. Differentiating iatrogenic hemorrhage and pathologic hemorrhage may not be possible, but findings to support pathologic hemorrhage may be present:
   1. RBCs within macrophages; however, in vitro erythrophagocytosis could occur within hours, so this should be assessed in freshly processed samples
   2. Hemosiderin within macrophages indicating pathologic hemorrhage from days to months ago
   3. Hematoidin (bilirubin) crystals, free or within macrophages
   4. Xanthochromia, though multiple causes must be considered (see Routine CSF Analysis, II.A.1)
   5. Pink CSF supernatant, although RBC hemolysis could occur in vitro

C. Pathologic hemorrhage may cause an increased TNCC, neutrophilic to mixed-cell inflammation, and increased CSF [protein].

D. Pathologic hemorrhage may occur with trauma, neoplasms, hemostatic defects, noninfectious inflammatory disease, and many infectious disorders (e.g., equine herpesvirus myeloencephalitis and thromboembolic meningoencephalitis in cattle infected with *Histophilus somni*).

VII. Neoplastic pleocytosis

A. Neoplasms within or affecting the CNS usually do not exfoliate cells into the CSF to cause neoplastic pleocytosis that can be detected with routine concentration techniques. However, neoplasms associated with the leptomeninges or ependymal lining (including choroid plexus) may, so cytologic evaluation of CSF is diagnostic in some cases.

B. Neoplastic cells that have been identified in CSF include lymphocytes (usually metastatic lymphoma, not primary CSF lymphoma), plasma cells, histiocytes (from primary CNS or disseminated histiocytic sarcoma), mast cells, choroid plexus cells (papillomas or carcinomas), oligodendroglioma cells, medulloblastoma cells, and metastatic carcinoma cells (leptomeningeal carcinomatosis).

C. These cells may or may not be accompanied by an increase in inflammatory cells. An increase in CSF [protein] is common.

VIII. Increased protein concentration without increased TNCC

A. Increased CSF [protein] without increased TNCC is a relatively common nonspecific abnormality that has been referred to as *albuminocytologic dissociation*, *protein–cytologic dissociation*, or *hyperproteinorrhachia without pleocytosis*. Albuminocytologic dissociation may be an appropriate category when the CSF [albumin] is specifically measured (as is done in human medicine but rarely in veterinary medicine).

B. Pathogenic mechanisms are the same as those provided for increased CSF [protein] (Table 21.5), but there is no concurrent stimulus for significant movement of inflammatory cells into the CSF at the time of sample collection. Inflammatory cells may be drawn to other parts of the CNS.

C. Causes include ischemic myelopathy (e.g., fibrocartilaginous embolism or cerebrovascular thrombi, especially when multiple), vasculitis (e.g., feline infectious peritonitis), trauma, degenerative myelopathies, myelomalacia, intervertebral disc herniation, seizures (e.g., idiopathic epilepsy), paroxysmal dyskinesia, brain or spinal cord neoplasms (especially of choroid or meninges, and protein may be very high caudal to an obstructive mass), acute canine polyradiculoneuritis (may be more evident with lumbar samples), inflammatory diseases (e.g., few dogs with idiopathic granulomatous meningoencephalitis, idiopathic encephalomyelitis in cats), cranial nerve neuropathy, and idiopathic vestibular disease.

IX. Other
- A. CSF analysis may yield [protein] and/or TNCC values that are marginally increased and accompanied by mild to moderate amounts of blood contamination without clear cytologic abnormalities of cell percentages or cells. Such findings are in the "grey zone" and suggest the possibility of disease without providing definitive evidence.
- B. Rarely, CSF collection at the cerebellomedullary cistern yields contents of an intracranial epidermoid cyst, a developmental defect caused by inclusion of ectodermal elements during neural tube closure. Cytologic preparations consist mostly of many anucleate squames and cholesterol crystals. These cysts may cause neural disease by compressing local tissue.
- C. Intracranial and spinal arachnoid cysts are developmental abnormalities that differ from epidermoid cysts. Reports often do not include CSF findings, but there may be an increased [protein] with little or no increase in TNCC, and mild hemorrhage has been present in some cases. If abnormal CSF findings accompany an arachnoid cyst, the cyst may or may not be the cause of them or of any detected neurologic deficits (could be an incidental finding).
- D. WBCs with cytoplasmic abnormal vacuolation or granulation (Table 2.19) may be present in CSF collected from young animals with neurologic signs because of a lysosomal storage disease (e.g., GM gangliosidosis, mucopolysaccharidosis). No other CSF abnormalities might be present. Blood smear examination may reveal the affected WBCs without the need for CSF analysis.

OTHER SELECTED ANALYSES

I. Protein fractions
- A. Protein electrophoresis
  1. Theoretically, concurrent protein electrophoresis of paired CSF and serum samples may be used to detect protein fractionation patterns and ratios that support blood–brain barrier leakage, intrathecal immunoglobulin production, or both. For example, an increase in the *albumin quotient (albumin index)*, the ratio of the CSF [albumin] to the serum [albumin], would support increased vascular permeability if the sample was collected with a "clean tap." However, neither diagnostic nor prognostic utility have been clearly demonstrated for protein fractionation in veterinary species with neurologic disorders,[62] and testing and reference values are not generally available.
  2. Agarose, cellulose acetate, or high resolution electrophoresis have been used, the first two methods often requiring substantial concentration of sample proteins to generate reliable electrophoretograms. When sample proteins must be concentrated, sample volume may be limiting, particularly for small animals, and especially if CSF [protein] is not increased or is minimally increased. Results of a study using membrane filtration to concentrate canine CSF samples for agarose gel electrophoresis suggest that 0.5–1.0 mL of CSF would generally be adequate initial volumes.[63] Other concentration methods may require unattainable volumes to begin with.
  3. Immunoelectrophoresis and isoelectric focusing with immunoblotting or immunofixation may prove helpful to evaluate intrathecal immunoglobulin production and help identify certain disorders.
- B. The Pandy test (developed by the Hungarian neurologist Pándy Kálmán)
  1. A quick, crude, qualitative procedure typically considered a test for CSF [globulin] but not specific for globulins[64] and largely replaced by more quantitative and objective protein assays

2. Addition of 50 µL of CSF to 1 mL of Pandy solution, a saturated aqueous solution of carbolic acid (phenol), promotes protein precipitation and turbidity, which is graded 1+ to 4+, immediately and after 15 min. A drop of serum added to 1 mL of Pandy solution can be used as a positive control.
3. Albumin at concentrations as low as 25 mg/dL, a mild increase for CSF, produced a small degree of turbidity immediately and after 15 min.[64]
4. Turbidity is evidence of increased permeability of the blood–CSF barrier or increased local immunoglobulin production.

C. The largely historical Nonne or Nonne–Apelt tests for CSF globulins include several crude, qualitative method variations based on the precipitation of globulins by ammonium sulfate in a saturated aqueous solution that is either mixed with an equal volume of CSF (often 1 mL) or overlaid with CSF. Turbidity in the mixed solution or at the interface of the solution and CSF sample is subjectively graded and interpreted as an increase in CSF [globulin]. In contrast to the Pandy test, albumin solutions of up to 100 mg/dL did not cause turbidity when mixed with equal volumes of saturated ammonium sulfate solution.[64]

II. Glucose concentration

A. The CNS requires glucose for energy (glycolysis, oxidative phosphorylation) and antioxidant generation (pentose phosphate shunt), and the source of CNS glucose is the blood. Glucose crosses the blood–brain barrier by facilitated diffusion mediated by glucose transporters, including glucose transporter protein type 1 (GLUT1).

B. CSF [glucose] can be measured by routine serum or plasma methods. Measurement is not routine in veterinary medicine as neither diagnostic nor prognostic utility have been clearly demonstrated at this time.

C. CSF [glucose] tends to reflect plasma [glucose] but is usually lower, reportedly averaging 40 % lower than serum [glucose] in people (lumbar taps),[15] 0–25 % lower in dogs (cerebellomedullary taps),[16] and 25 % lower in cats (cerebellomedullary taps).[16] In dogs, CSF [glucose] changed within minutes of increases or decreases in plasma [glucose] induced by insulin or intravenous glucose administration, with incremental changes in CSF [glucose] lagging behind those of serum [glucose] by about 5–40 min, depending on the dog and the rate and direction of change.[16] The lag time for equilibration is expected to be greater for lumbar samples, and equilibration may not occur in cases of marked hyperglycemia. Llama CSF [glucose] was reported to be about 60 % lower than serum [glucose],[14] but this greater difference from other species may have been related to epinephrine- or xylazine-induced increases in serum [glucose] without time for CSF glucose equilibration.

D. Hyperglycorrhachia occurs with hyperglycemia, which promotes facilitated diffusion of glucose into the CNS. Other causes are acute hemorrhage, blood contamination during CSF collection, and increased blood–brain barrier permeability caused by inflammation.

E. Hypoglycorrhachia may occur with infectious or noninfectious inflammatory conditions or neoplasms in the CNS. Contributing mechanisms are decreased glucose transport through the blood–brain barrier and increased glucose consumption by resident cells, inflammatory cells, neoplastic cells, and organisms. Increased CSF [L-lactate] is expected in these conditions. Glucose transporter protein type 1 (GLUT1) deficiency in people causes hypoglycorrhachia associated with developmental delays and seizures in infancy and early childhood; CSF [L-lactate] is typically not increased.[65,66]

F. CSF [glucose] may be the net result of opposing factors promoting both hyperglycorrhachia and hypoglycorrhachia, and because hyperglycemia is the main cause of hyperglycorrhachia, use of a $[glucose]_{CSF}$:$[glucose]_{serum}$ ratio may help detect pathologic processes promoting hypoglycorrhachia.

III. L-Lactate concentration (see Chapter 9 for additional L-lactate information)

A. Lactate is abundant in the CNS and an important energy substrate for neurons, the cells that use most of the energy consumed by the brain. Most of the CNS lactate is produced within the CNS, and the major cell source appears to be astrocytes.[67]

1. Astrocytes generate L-lactate from glucose by aerobic glycolysis (glycolysis without hypoxia), and they generate even more L-lactate through anaerobic glycolysis when hypoxia is present. The generated L-lactate exits astrocytes and enters neurons through monocarboxylate transporters, and neurons convert the lactate to pyruvate, which leads to energy and intermediate metabolite generation via the Krebs cycle and oxidative phosphorylation.
2. Some L-lactate enters the CNS from blood by facilitated diffusion through endothelial cell monocarboxylate transporters. This uptake can increase in hyperlactatemic states.

B. CSF [L-lactate] can be measured by methods used routinely for whole blood or plasma. Point-of-care options are available, but assay precision and accuracy vary among analyzers. Clinical samples are best analyzed promptly (within 60 min) to avoid potential in vitro increases from L-lactate generation by cells. Some studies show little change in [L-lactate] with storage of individual or pooled samples for at least 24 h at 4 °C or room temperature, but stability studies of individual samples with known high TNCCs were not found. Options for delayed analysis include frozen aliquots or use of blood tubes containing sodium fluoride to inhibit glycolysis.

C. Appropriate analyzer-specific and population-specific RIs from adequate numbers of healthy individuals are generally not available, and assay variation limits the utility of universal medical decision limits.[68] However, [L-lactate] in CSF samples from dogs with no imaging or routine CSF analysis abnormalities provide some basis for comparison. Collection site may influence [L-lactate], with mildly greater concentrations in lumbar samples compared to cerebellomedullary samples.

D. Increases in CSF [L-lactate] relate mostly to increased L-lactate formation from increased CNS glycolysis. Potential causes vary from specific genetic defects in metabolic pathways to more generalized tissue hypoxia. Additionally, glycolysis is the dominant energy-production pathway in many neoplastic cells, even under aerobic conditions (aerobic glycolysis), so many neoplasms generate increased amounts of L-lactate. L-Lactate from organisms, WBCs, and RBCs may also contribute, including with iatrogenic hemorrhage and delayed analysis. Weak to moderate correlations of [L-lactate] with TNCC and nucleated cell fractions have been reported.[69,70]

E. More specifically, increases in CSF [L-lactate] of domestic mammals have been documented with inflammatory, neoplastic, and hemorrhagic disease. CSF L-lactate concentrations overlap considerably among various groups of disorders, so increases are nonspecific and must be interpreted with consideration of all clinical and laboratory findings including diagnostic imaging, routine CSF analysis, and often tests for evidence of infectious agents.

1. Inflammatory CNS disease
   a. Increased [L-lactate] was present in approximately 50 % of dogs with inflammatory CNS disease and was not useful to differentiate inflammatory cell populations, disorders, or outcomes.[69] Inflammatory diseases included idiopathic granulomatous meningoencephalitis, idiopathic necrotizing meningoencephalitides, steroid-responsive meningitis-arteritis, and infectious or presumed infectious meningoencephalitis or meningomyelitis.
   b. Increased [L-lactate] was present in many cattle with viral (rabies, herpesvirus 5) or bacterial meningoencephalitis, and although overall increases were greatest with bacterial infections, the magnitude of increase did not differentiate the disorders.[70]

2. CNS neoplasms may increase CSF [L-lactate] directly through increased glycolysis and L-lactate production and secretion, or indirectly through impaired circulation, inflammation, necrosis, or hemorrhage.
3. Seizures: Seizure activity appears to have minimal effect on CSF [L-lactate] in dogs, increases being related more to underlying disorders.[71] Some dogs with seizures from inflammatory, neoplastic, or vascular disease had increased CSF [L-lactate], but dogs with idiopathic epilepsy or seizures related to toxicants or metabolic disease had little or no increases. Increases have been attributed to seizure activity in people.
4. Hemorrhage: CSF [L-lactate] increased after introduction of 2–3 mL of autologous blood to mimic intracerebral or subarachnoid hemorrhage in dogs. Increases were greater with the former, supporting that increased production by brain cells contributed L-lactate in addition to any L-lactate derived from blood cell metabolism.[72]
5. Other: Ischemic vascular disease, traumatic injury with or without hemorrhage, and any of several genetic metabolic disorders recognized in people could cause increases in CSF [L-lactate].

IV. Enzyme activities
- A. CSF activities of creatine kinase (CK) and various other enzymes (e.g., aspartate transaminase, alkaline phosphatase, lactate dehydrogenase, and matrix metalloproteinases 2 and 9) have been or are being explored for diagnostic or prognostic use in various veterinary species, but little clinical utility has been clearly demonstrated.
- B. Creatine kinase (CK) activity in CSF
  1. In people, CNS CK is present as the mitochondrial isoenzyme in neurons and as the CK-BB isoenzyme (CK-1) in selected neurons and astrocytes (glial cells).[73] The latter predominates in blood-free human CSF.[74] In dogs, CK-BB and mitochondrial CK activities were present to various degrees throughout the brain, typically in a 10:1 ratio or greater and without CK-MB or CK-MM.[75]
  2. Damage to CK-expressing neurons and glial cells, particularly those juxtaposed to the CSF, may increase total CSF CK activity. Assays with isoenzyme specificity, if available, would further characterize the increase.
  3. In veterinary medicine, CSF CK activity can be measured with routine serum or plasma CK assays. Results vary with different assay methods, including different reaction temperatures. Results from some healthy dogs may be below the analytical range of the assay, but only increases are of interest.
  4. CSF CK stability: CK activities in canine CSF that was frozen at −80 °C for 17–78 d did not agree with fresh activities.[76] In another study, reported data suggest that CK activity in canine CSF samples frozen at −4 °C may decrease after 1 wk of storage, and decreases in activity may occur by 24 h of storage at 25 °C.[77] When possible, CK analysis should be done on a fresh sample, as conditions that alter CK stability are not established.
  5. Collection site may influence CSF CK activities, with greater activity nearer to or caudal to a lesion. CK activity was greater in lumbar cisternal samples than in paired cerebellomedullary cisternal samples collected from dogs with thoracolumbar myelopathies.[78]
  6. Increased CSF CK activity
     a. Increases can occur with blood contamination of the sample because of plasma CK activity and potentially because of interference from RBC content.
     b. Increases have been reported in a variety of CNS disorders of domestic mammals, but without enough diagnostic utility to warrant routine testing.

c. CSF CK activity may be a prognostic marker in some conditions, such as intervertebral disk herniation in dogs, but it is important to recognize that a decision threshold for one CK assay may not apply to other assays.[76]

V. Tests for microorganisms: Microorganisms may be present in the CNS without being in the CSF, or they may be present in CSF but not seen in cytologic preparations of CSF because of low numbers or low recovery. When suspected based on clinical or laboratory findings, microorganisms may be further assessed by culture, polymerase chain reaction tests, or both. Antibody titers in CSF, serum, or both may be used to help diagnose certain infectious neurologic diseases. High antibody titers to particular organisms could be from vaccination, exposure without disease, or disease, and must be interpreted accordingly. Testing acute and convalescent samples in the same assay run generally provides the most valuable information. Even slight blood contamination of CSF can transfer blood antibodies to CSF samples and impair reliable measurement of CSF titers.[17] Further information about testing for microorganisms causing neurologic disease is available in neurology and infectious disease resources.

References

A complete set of references is available online. Please use the below link to access the References files.

https://www.wiley.com/go/fundamentals/vet

Appendix A

THE COMPLETE BLOOD COUNT AND BLOOD SMEAR EVALUATION

I. Overview of a complete blood count (CBC), also known as a full blood count (FBC), full blood exam (FBE), or hemogram
   A. Numerical (quantitative) and microscopic (quantitative and qualitative) data are components of mammalian CBC results. The primary purposes of the CBC are to screen the hemic system for abnormalities or response to disease, and to confirm or define the presence of a hematologic disorder.
   B. Major components of the CBC
      1. *Leukogram* (meaning "leukocyte picture"): results of laboratory assays and calculated data that characterize the white blood cells (WBCs) in blood (see Chapter 2)
      2. *Erythrogram* (meaning "erythrocyte picture"): results of laboratory assays and calculated data that characterize the red blood cells (RBCs) in blood (see Chapter 3)
      3. *Thrombogram* (meaning "platelet picture"): results of laboratory assays and calculated data that characterize the platelets in blood (see Chapter 4)
   C. Basic information obtained from the results of a CBC
      1. If a CBC result is within reference interval, the net effect of a disease on that aspect of the hematopoietic system has been minimal.
      2. If a cell concentration is increased, the disease caused at least one of the following:
         a. Increased production of that cell type
         b. A shift of that cell type from a storage or noncirculating pool to circulating blood
         c. An increased circulating life span of the cell type because the rate of cell loss to tissues or rate of cell death is decreased
      3. If a cell concentration is decreased, the disease caused at least one of the following:
         a. Decreased production of that cell type
         b. A shift of that cell type from the circulation to a noncirculating pool
         c. Decreased circulating life span of the cell type
      4. If morphologic features of a given cell type are abnormal, either (i) a defect in hematopoiesis caused production of abnormal cells, or (ii) morphologic abnormalities were acquired as the cells circulated in the body.
   D. Blood sample
      1. For domestic mammals, blood is collected into a tube containing EDTA and immediately mixed by slowly inverting the tube at least eight times (over and back is one inversion). Anticoagulation is needed for complete and accurate results. Samples must be free of clots and platelet clumps because cells trapped in clots are not counted, platelets trapped in platelet clumps are not counted, and platelet clumps may alter platelet indices and be counted as WBCs.
      2. Stability of cells: Cell concentrations are generally stable for ~8 h at room temperature and ~24 h with refrigeration, but cell changes begin immediately after collection and progress over hours and days, leading to unreliable results for many quantitative and qualitative

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assessments, e.g., RBC indices, reticulocyte concentration, platelet concentration, WBC differential count, toxic change, and poikilocytic RBCs.

E. Quantitative analysis with a hematology analyzer
   1. Many types of hematology analyzers provide useful data for domestic mammals, but results vary among analyzers and appropriate reference intervals should be used.
   2. Manufacturer instructions for use and maintenance should be followed.
   3. Control samples, when available, should be used to provide evidence of appropriate testing. Commercial laboratories run controls daily or each shift.
   4. All analyzers are subject to error, particularly with various types of hematologic abnormalities. Recommendations provided for any flagged data should be followed (e.g., repeating analysis, evaluating equipment, or examining a blood film).

F. Blood film stains
   1. Air-dried blood films are stained with a Romanowsky-type stain that provides differential staining of cells. *Romanowsky stains* were described by Romanowsky (1891) as a combination of eosin and methylene blue that produced a spectrum of colors from blue to reddish orange, depending on the pH of the cell's content. *Wright stain* is a combination of eosin and oxidized methylene blue, but "Wright stain" often refers to a Romanowsky-type stain and not the original Wright stain. Oxidized methylene blue stains are called *azure dyes*; Romanowsky stains made with combinations of azure dyes and eosin include Giemsa, Wright-Giemsa, Wright-Leishman, and Diff-Quik stains. Diff-Quik involves methanol fixation but uses aqueous-based eosin and azure dyes that can remove water-soluble material including content of mast cell and some basophil granules.
      a. Acidic structures (e.g., DNA and RNA) attract the basic azure dyes, which stain structures various colors from purple to blue.
      b. Alkaline structures (e.g., hemoglobin and eosinophil granules) attract the acidic eosin dye and stain structures from red or pink to orange.
      c. Terms used to describe colors or staining properties
         (1) *Neutrophilic*: *neutro* (neither alkaline nor acidic) plus *philic* ("loving")
         (2) *Eosinophilic*: loves eosin (acidic dye), so stains red to orange; use of a specific color (e.g., orange, pink, magenta) is more descriptive than *eosinophilic*
         (3) *Basophilic*: loves basic (alkaline and azure) dyes, so stains blue to purple; use of a specific color (e.g., deep blue, grey-blue, purple) is more descriptive than *basophilic*
         (4) *Azurophilic*: loves azure dye, so takes up blue to purple stain

G. Microscopic examination of a stained blood film
   1. This is best included as part of every CBC, but it is particularly important for sick patients and those with abnormal concentrations of WBCs, RBCs, or platelets. Abnormalities are discussed in Chapters 2–4.
   2. A blood film is made to obtain an even distribution of cells and an adequate *optimal zone* or "counting window," that part of the smear where RBCs occasionally touch each other and where nuclear and cytoplasmic features of WBCs are distinct because the cells are well flattened like fried eggs. This area is commonly called a monolayer, but cells can be in a single layer (a monolayer) without being well spread or in the optimal zone where they are best assessed. The optimal zone is generally ~1–2 10× field diameters into the body of a blood film from the feathered edge (varies with the technique and sample). RBC density does not define the optimal zone; RBCs are more widely separated in the optimal zone of blood films from anemic individuals.

3. Blood films are examined at low magnification with 4× or 10× objectives, at higher magnifications with high-dry (40× or 45×) or oil objectives (50× or 60×), and with a 100×-oil objective. The following questions are addressed.
   a. Are the cells evenly distributed and properly stained?
   b. Are there abnormal large structures in the blood (often concentrated in the feathered edge) such as microfilariae, platelet clumps, macrophages, or epithelial cells? If so, record their presence. Endothelial cells (from venipuncture) and megakaryocytes are rarely present and not pathologic.
   c. Do the RBC and WBC densities correspond with the known cell concentrations in the sample? If not, check the accuracy of the cell concentrations. If cell concentrations are not available, do cell concentrations appear decreased or increased?
   d. Does an estimate of the platelet density align with the expected values based on analyzer data or health? Are there giant or shift platelets (platelets wider than normal RBCs) or platelet inclusions? Record any abnormalities.
   e. Do RBCs have abnormal shapes, sizes, colors, inclusions, or associated organisms? Evaluate the RBCs and record abnormalities.
   f. What types and proportions of WBCs are present? Complete a WBC differential count and record it. Use it with the analyzer [WBC] to calculate concentrations of each WBC type. Evaluate the observed WBCs for morphologic abnormalities and record the findings.

II. One step-by-step approach to blood smear evaluation (this can be abbreviated for efficient assessment of major or specific abnormalities in the clinical setting)
   A. Verify that the slide label matches the appropriate patient.
   B. Use a well-maintained microscope of adequate quality adjusted for appropriate (near Köhler) illumination.
   C. Examine at low magnification: 4× and 10× objectives.
      1. Scan representative parts of the film, including the feathered edge (a good place to begin), optimal zone, and deeper portions. If staining is poor, re-stain. A new smear may be indicated if (i) the feathered edge extends beyond the stained region, (ii) there is excessive stain precipitate or other artifact, (iii) WBCs are disproportionately concentrated in the feathered edge (occurs with some smearing techniques), (iv) there is little or no optimal zone for morphologic assessment of cells, or (v) WBCs are badly distorted or damaged (if a high Hct is contributing, smears can be made after diluting the blood, e.g., 1:1 with phosphate-buffered saline, ideally containing stabilizing albumin).
      2. Note the presence of any microfilariae, unexpected cells, or platelet clumps (see Figure 4.4 for platelet clumps and Figure A1 for microfilariae).
      3. Check for even distribution of WBCs; report leukoagglutination if present. If it is moderate or marked, the [WBC] and WBC differential may be inaccurate.
      4. Roughly estimate the [WBC] (clearly low, clearly high, similar to health) to check the automated [WBC]. If a mismatch is suspected, rerun the sample through the analyzer, make a new blood smear, and reassess.
      5. Assess for rouleaux and agglutination (Figure 3.7).
         a. Rouleaux should be reported if increased for the species. When rouleaux extend to the feathered edge of a blood film, rouleaux should be reported in all species.
         b. When agglutination is severe, the base of the film may appear thinner and the feathered end of the film thicker (Figure 3.7D). Agglutination is best assessed in the middle or thicker portions of a blood film. Be very cautious concluding that

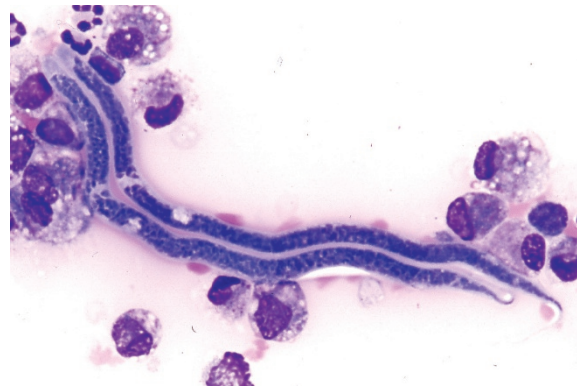


Figure A1. Two microfilariae of *Dirofilaria immitis* at the feathered edge of blood film from a dog with heartworm disease.

agglutination is present when rouleaux are prominent. If agglutination is present, most of an analyzer's RBC data may be inaccurate.

D. Examine WBCs in the optimal zone of the slide at high magnification: 40-60× and 100× objectives.
   1. Perform a microscopic WBC differential count if analyzer data are unavailable, if analyzer flags indicate unreliable WBC data, if analyzer histograms suggest the potential for unreliable data, or if any of the following are present on the smear: several (i) atypical WBCs, (ii) immature neutrophils, (iii) nRBCs, (iv) eosinophils with nonstaining granules (or not detected by an analyzer), or (v) basophils. The starting field for the WBC differential should be standard, not chosen to include any specific cell type.
      a. Differentiate and count intact segmented neutrophils, nonsegmented neutrophils (identify all stages separately), lymphocytes, monocytes, eosinophils, basophils, mast cells, and any other cell types. Classify atypical cell populations of uncertain identity as "other" or "blast" cells. Cells with a lymphoid appearance may be immature nonlymphoid hemic cells, e.g., in acute myeloid leukemias, making "other" or "blast" more appropriate classifications than "lymphocyte" in such cases.
      b. Differentiate a minimum of 100 WBCs unless prevented by marked leukopenia, in which case record the lower number of cells assessed (alternatively, use multiple smears to differentiate 100 WBCs). When WBC concentrations are increased, differentiating greater numbers of WBCs increases precision and accuracy of WBC differential counts (see Table 2.3). One approach is to differentiate 200 WBCs if the [WBC] is 20,000–29,999/μL and 300 WBCs for WBC concentrations ≥ 30,000/μL.
      c. Include only intact cells within the optimal zone, with the differential count progressing in a battlement pattern from thinner to thicker areas and back again across the optimal zone (Figure A2). Do not extend so far into the feathered edge or into thick regions of the smear that cells tend to be lysed (scrambled eggs) or too balled up (poached eggs) to identify reliably.
      d. Record when there is an excessive number of lysed cells, e.g., ≥ 20 %; the WBC differential may be less reliable than usual.



Figure A2. A recommended battlement approach to WBC differential counts. By including thinner and thicker regions of the optimal zone rather than moving directly across the slide, this pattern reduces bias caused by larger cells having the tendency to move further toward the end of the smear.

e. Differentiating segmented neutrophils (segs) from band neutrophils (bands):
   (1) To classify neutrophils as bands or segs, assess: (i) the width of the nucleus, (ii) consistency of the nuclear width, and (iii) the degree of chromatin clumping and staining intensity. Each of these forms a continuum and none alone can identify a band. Segmented neutrophils typically have narrow nuclei with focal indentations (lobe formation) and clumped, dark purple chromatin, whereas features of band neutrophils are:
      (a) Relatively parallel nuclear margins (C, S, or U shape) without focal narrowing of > 1/3 the nuclear width in the region of narrowing
      (b) Increased nuclear width compared to mature neutrophils
      (c) Less clumped and paler staining chromatin than in mature neutrophils
      (d) Relatively smooth nuclear margins, but small knobs or thorns may be present, particularly in equine bands
   (2) Most classification differences occur with seg-band "inbetweeners" or with asynchronous maturation from toxic change, but inbetweeners are usually a small minority of the neutrophils, and toxic change, like a left shift, is itself an indication of inflammation. The following may help classify neutrophils that do not have classic features of bands or segs.
      (a) If a neutrophil nucleus overlaps itself, that does not mean there is a narrowing in the nucleus. It is reasonable to classify a cell as a band or seg based on the above features of the visible portions of the nucleus.
      (b) If the nucleus is mostly wide, pale, and smooth but has a focal narrowing > 1/3 the nuclear width in the region of the narrowing (whether indented on one side or both), the cell has immature features but is segmented and not a band. This asynchronous maturation can occur with inflammation.
      (c) If a nucleus has pale chromatin with relatively smooth margins, and is broader at the ends with gradual tapering toward the middle, the cell has immature features and may best be classified as a band even if the width of the nucleus in the middle is less than two-thirds the width at the ends (no focal indentation).
      (d) If neutrophils appear immature but do not meet criteria for classification as bands, they may be reported as hyposegmented or mildly immature cells and interpreted as evidence of early release and inflammation.
      (e) Do not report neutrophils with hyposegmented nuclei and mature chromatin patterns seen with Pelger–Huët anomaly or similar disorders as band neutrophils, because they do not reflect immaturity.

f. Differentiating band neutrophils from more-immature forms (typically not expected without an increase in bands)
   (1) A metamyelocyte nucleus is an indented oval (kidney bean shaped); the nucleus of a band neutrophil is more elongate. Metamyelocyte cytoplasm is like that of a band neutrophil.
   (2) A myelocyte has a round to oval nucleus. The cytoplasm is like that of band neutrophils and metamyelocytes.

2. Evaluate WBCs for morphologic abnormalities.
   a. Neutrophil toxic change
      (1) Döhle bodies are rare or absent from neutrophils of healthy domestic mammals other than cats. Therefore, except in cats, it is reasonable to report toxic change when ≥ 2 neutrophils have Döhle bodies or ≥ 2 segmented neutrophils have diffuse basophilia (excluding stain variation when all neutrophils are basophilic and lack other features of toxic change). In cats, one in-house study suggested that toxic change is present when at least half the neutrophils contain Döhle bodies or when at least one-third of the neutrophils with Döhle bodies contain at least three Döhle bodies.
      (2) Other toxic changes, including foamy cytoplasm, giant neutrophils, ring nuclei, or prominent (retained) primary granules, may also be present; consider other causes for these changes if Döhle bodies are absent. Clear, sharp, cytoplasmic vacuoles occur following exposure to EDTA and are not indicative of toxic change. Immature neutrophils (e.g., bands, metamyelocytes) may have diffuse cytoplasmic basophilia (not Döhle bodies) because of immaturity.
      (3) When toxic neutrophils are large and have blue cytoplasm and immature nuclei, they may be difficult or impossible to differentiate from monocytes. Such toxic change is severe. See Chapter 2, Abnormal Morphologic Features of WBCs, section I.A.4 for information about grading toxic change.
      (4) Note: in vitro changes mimicking toxic change can occur in old samples. This often appears as one or two punctate (tiny and round) blue Döhle-like bodies in neutrophils unaccompanied by other toxic changes like diffuse basophilia and foaminess (Figure 2.12).
   b. Reactive or atypical lymphocytes
      (1) Reactive lymphocytes occur in health, particularly in young animals, but they can become more prevalent with inflammation. They have increased cytoplasmic basophilia and often increased cell size and lobulated nuclei (Figure 2.13). Granular lymphocytes and lymphocytes with increased amounts of pale cytoplasm are not reactive lymphocytes, although they might be part of an inflammatory response. It is reasonable to record an increase in reactive lymphocytes when they account for at least 10 % of the lymphocytes and lymphocytosis is present, or when at least 20 % of the lymphocytes are reactive and lymphocytosis is not present. When reactive lymphocytes are that prevalent, clinically significant inflammatory disease is likely.
      (2) Atypical (i.e., not typical) lymphocytes may be either reactive lymphocytes or neoplastic lymphocytes. An individual neoplastic lymphocyte can look identical to a reactive lymphocyte, but neoplastic lymphocytes can be distinguished from reactive lymphocytes if they form a large monotypic population or have certain features not expected in nonneoplastic lymphocytes (requires expertise). Features of lymphocyte atypia include increased cell or nuclear size, cytoplasmic

basophilia, abnormal cytoplasmic granules, pleomorphic nuclear shape, immature chromatin pattern, and prominent nucleoli.

c. Infectious agents: Although rare in most locations, organisms that may be present within WBCs and that are diagnostic of disease include bacteria (e.g., rods including nonstaining *Mycobacterium* spp., cocci, *Ehrlichia* spp., *Anaplasma* spp.), yeast (e.g., *Histoplasma capsulatum)*, and protozoa. Distemper viral inclusions may also be present.

d. Noninfectious inclusions: These uncommon to rare inclusions include RBCs, hemosiderin (sideroleukocytes), lipofuscin, mast cell granules, and atypical granules or vacuoles associated with congenital disorders, e.g., lysosomal storage diseases, granulation anomaly in Birman cats (not a clinical problem), and Chédiak-Higashi syndrome.

3. If enough nRBCs were detected to potentially alter interpretation of the [WBC] or specific WBC concentrations, e.g., ≥ 10 nRBCs/100WBCs, correct the [WBC] for nRBCs (see Equation 2.1). Multiply the corrected [WBC] by the percentage of each WBC type to calculate the concentrations of each cell type.

E. Evaluate platelets: 100× objective.

1. Check for platelet clumps if they were not seen at lower magnification (Figure 4.4). If platelet clumping is absent, report that no platelet clumps were seen and interpret the [platelet] with respect to species-specific reference intervals. When platelets are clumped enough to report, perhaps > 2 tight platelet clumps of at least 10 platelets each, record the presence of clumped platelets and consider the analyzer [platelet] unreliable and likely an underestimation, particular with more and larger platelet clumps. If platelet clumping is mild and few platelets are present, thrombocytopenia may be present; verification requires a new blood sample without platelet clumping (atraumatic venipuncture with rapid anticoagulation).

2. When platelets are well distributed and not clumped, the [platelet] is estimated to check for gross errors in the analyzer [platelet] or to estimate a [platelet] in the absence of analyzer data. Several methods have been proposed, some requiring a [RBC] or [WBC] to calculate [platelet] based on the ratio of platelets to RBCs or WBCs. For the method provided here, assessment should be done in the optimal zone of the smear where WBCs are well flattened (fried-egg appearance). If done in thicker regions, as occurs with samples from anemic animals when fields are chosen to normalize RBC density, overestimation occurs even if the cells are in a monolayer, because the volume of fluid per field is greater in these thicker areas than in the optimal zone.

a. To estimate the [platelet], determine the mean number of platelets per 100×-oil objective field based on at least 3 fields (5–10 fields is better). When in the optimal zone of a good blood film, 1 platelet/field is equivalent to ~15,000/μL when using a microscope with widefield 10× oculars (field number 22). For example, if the mean number of platelets per field was 4, then the estimated [platelet] would be about 4 × 15,000/μL = 60,000/μL. If using regular rather than widefield oculars, a 20,000/μL factor would be more appropriate than 15,000/μL.

b. If a microscopic estimate differs substantially from an analyzer [platelet], e.g., by more than ~75,000/μL up to an analyzer concentration of 150,000/μL or by more than 75,000–100,000/μL for analyzer values of 150,000–250,000/μL, consider potential causes: (i) unrecognized platelet clumping, (ii) many large platelets not counted as platelets by the analyzer, (iii) many cell fragments or other particles (e.g., ghost cells, lipid droplets) counted as platelets by the analyzer, or (iv) an inaccurate

microscopic estimate for any of several reasons. Interpret accordingly. Discrepancies at greater concentrations are of less concern, and margins of error are greater, e.g., ± 200,000–250,000/μL.

c. Except in horses and certain dog breeds (e.g., greyhounds) that have lower platelet concentrations in health, thrombocytopenia is likely with < 8 platelets/field and very likely with < 5 platelets/field (100× oil, widefield oculars).

3. In the absence of a standardized approach to reporting large platelets, it is reasonable to report the presence of large platelets when at least 3 are seen over several fields. Platelets can be considered *large* when their diameter is greater than the diameter of a normal-sized RBC (Figure 4.5). If many platelets are large and therefore not recognized as platelets, the analyzer [platelet] and MPV may be falsely decreased.
4. Report any other platelet abnormalities (rare) (Figure 4.5F).

F. Evaluate RBCs for morphologic abnormalities: 100× objective.

1. Examine RBCs for abnormalities of size, shape, color, organisms, inclusions other than organisms, and again for rouleaux and agglutination if low-magnification evaluation was insufficient. When grading abnormalities, a 3-level scale (mild or 1+, moderate or 2+, and marked or 3+) is probably more reproducible than a 4-level scale (1+ to 4+) and is sufficient for diagnostic use. An example is provided (Table A1).
2. Color: Abnormalities of color include polychromasia, hypochromasia, apparent hyperchromasia, and ghost cells (Figures 3.9–3.11 and 3.15–3.16). Although spherocytes and pyknocytes appear hyperchromic and ghost cells are extremely

Table A1. Grading of RBC abnormalities for dogs, cats, horses, and cattle[a]

	Mild	Moderate	Marked
	mean number per 1000× field		
Polychromasia			
Dogs	2–5	6–12	> 12
Cats and cattle	1–3	4–10	> 10
Hypochromasia, spherocytosis, microcytosis, macrocytosis, unclassified poikilocytosis	1–13	14–65	> 65
Acanthocytosis, dacryocytosis, drepanocytosis, eccentrocytosis, pyknocytosis, schistocytosis, ghost cells	1–3	4–10	> 10
Codocytosis	3–7	8–20	> 20
Echinocytosis	5–19	20–100	> 100
Elliptocytosis	2–4	5–10	> 10
Keratocytosis	3–5	6–10	> 10

[a] Grading scales for llama and alpaca RBC abnormalities were not found; for polychromasia, the cat and cattle scale may be appropriate, and the other scales may be appropriate for the other changes most often seen in these species: hypochromasia, microcytosis, dacryocytosis, and drepanocytosis.

Note: This table is a modification of published 4-level scales based on microscopic fields with normalized RBC densities[1] and of scales developed at the Veterinary Diagnostic Laboratory, Michigan State University, based on cell assessment in the optimal zone. Values incorporate experience and limited data; they should not be considered definitive.

hypochromic, these cell types are reported specifically as spherocytes, pyknocytes, or ghost cells rather than as hyperchromic or hypochromic cells.

a. Polychromasia should be reported and graded when increased for the species.
   (1) In one approach, polychromasia is assessed in fields with a normalized RBC density defined by approximately half of the RBCs touching another.[1] With an anemic patient, a field with half of the RBCs touching each other would be thicker than a field in the optimal zone, and it would represent a greater blood volume. This method is analogous to determining a polychromatophil percentage, like a reticulocyte percentage, so an increase could be caused by a decrease in mature cells rather than an increase in polychromatophils.
   (2) Alternatively, polychromatophils can be counted in the optimal zone where the RBC density is decreased in the presence of anemia but the blood volume per field remains more uniform; with this approach, grading is more a reflection of concentration and less of percentage (see Table A1).
   (3) Polychromatophil concentrations correlate with analyzer reticulocyte concentrations, so moderate and marked discrepancies should be investigated (e.g., moderate analyzer reticulocytosis with no increase in polychromasia). The [reticulocyte] generated by a hematology analyzer is a more quantitative and objective assessment of reticulocytosis and erythropoiesis, but false increases and false decreases can occur.

b. Hypochromasia is reflected quantitatively by decreased mean cell hemoglobin concentration (MCHC), but MCHC may be decreased when visual hypochromasia is not present, e.g., with reticulocytosis, because reticulocytes have decreased cell hemoglobin concentrations due to excessive volumes, not decreased hemoglobin content, and generally do not spread thin enough on smears to appear hypochromic. Hypochromic cells should be differentiated from torocytes, which are artifactual poikilocytes with distinct, well-demarcated, punched-out centers (Figure 3.16H). Hypochromic cells have increased central pallor with a more gradual transition from peripheral pinkish-orange cytoplasm to central pallor, or, in camelids, nonhomogeneous hemoglobin distribution (Figure 3.10B and C). Interindividual agreement for detecting mild hypochromasia is probably poor, but MCHC should indicate its presence. See Table A1 for grading visual hypochromasia.

3. Size:
   a. Microscopically, increased variation in RBC diameters can be considered anisocytosis, but this microscopic anisocytosis does not align completely with variations in RDW, an objective assessment of RBC volume variation. For example, spherocytes appear small based on microscopic cell diameters, but their volumes are typically not significantly decreased; they are just unable to flatten on the slide. In contrast, hypochromic microcytes may flatten excessively to yield widths similar to those of normocytes. Recognizing the cause of apparent anisocytosis (i.e., increased macrocytes, increased microcytes, or increased poikilocytes such as spherocytes) is of more diagnostic value than simply identifying anisocytosis. Although poikilocytes can be recognized, it can be difficult to determine if anisocytosis is caused by cells with increased size or with decreased size based on microscopy.
   b. If MCV and RBC volume histograms clarify that there is an increase in large cells, small cells, or both, macrocytes or microcytes sometimes can be specifically graded (see Table A1), but this is different from anisocytosis (cells could be uniformly macrocytes or microcytes without anisocytosis). Without knowing if cells are

macrocytes or microcytes, apparent anisocytosis can be subjectively graded as mild, moderate, or marked based on the degree of apparent size variation combined with the proportion of cells that are not of uniform diameter. Semiquantitative grading of anisocytosis has been proposed based on "the number of variable-sized cells" per field,[1] but this does not include the degree of difference (largest versus smallest), and it is not clear which cells would be considered the "variable-sized" cells.

4. Shape: Poikilocytosis (an increase in poikilocytes from what is expected in health) should be reported and graded for each specific type (Figures 3.10, 3.15, and 3.16), but if shape abnormalities do not fit into any specific poikilocyte classification, they can be recorded as "unclassifiable" or "unclassified" poikilocytosis. As for polychromasia, poikilocytes can be graded based on counts in the optimal zone (see Table A1) or in fields with normalized RBC density.[1] With the latter method, mild increases may be caused by anemia, not by increased concentrations of poikilocytes. Lipemia may alter RBC appearance, cause RBC lysis, and interfere with assessment of RBC features, in which case the presence of lipemia should be noted.
5. Infectious agents (Figure 3.12): Organisms that may be present within or on RBCs include *Babesia* spp., *Mycoplasma* spp., *Anaplasma marginale*, *Cytauxzoon felis*, and *Theileria* spp. Distemper virus inclusions reflect an acute viral infection. The presence of organisms and distemper inclusions should be reported and confirmed by a veterinary hematologist/pathologist.
6. Noninfectious inclusions (Figure 3.13): RBC inclusions other than organisms and viral components include Howell-Jolly bodies, Heinz bodies, basophilic stippling, siderotic inclusions, and hemoglobin crystals.
   a. If Heinz bodies are seen or suspected in a Wright-stained blood smear, unless clearly in fewer than ~5 % of RBCs in cats, use a vital stain (e.g., new methylene blue) to confirm their presence and estimate their numbers as few (e.g., < 5 %), moderate, or many (e.g., > 50 %). Depending on the analyzer, moderate or many Heinz bodies may affect the [WBC], [lymphocyte], [hemoglobin], MCH, or MCHC.
   b. Howell-Jolly bodies are more prevalent in the blood of cats compared to other species because circulation through the feline spleen does not require RBCs to squeeze through endothelial cell slit pores as in other species, and consequently, fewer inclusions are "pitted out." For cats, a mean of at least one Howell-Jolly body per 100×-objective field is probably needed to report them as increased. In other species, a mean of at least one Howell-Jolly body in every four to five fields is probably enough to report them as increased.
   c. Basophilic stippling and siderotic inclusions are not expected in health. It is reasonable to report either of them when at least two or three of them are found during review of the blood smear.
   d. Hemoglobin crystals are rarely of clear diagnostic value but should be recorded subjectively (few to many). They are common and of no diagnostic significance in camelid blood smears, and they can develop in stored blood from other species.

G. For any blood film with uncertain findings that may be of diagnostic importance, review of the blood film by an expert veterinary hematologist/pathologist may be valuable, as may submission of a fresh blood sample for a timely CBC and expert evaluation.

Reference

1 Weiss, D.J. (1984). Uniform evaluation and semiquantitative reporting of hematologic data in veterinary laboratories. *Vet Clin Pathol* 13:27–31.

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