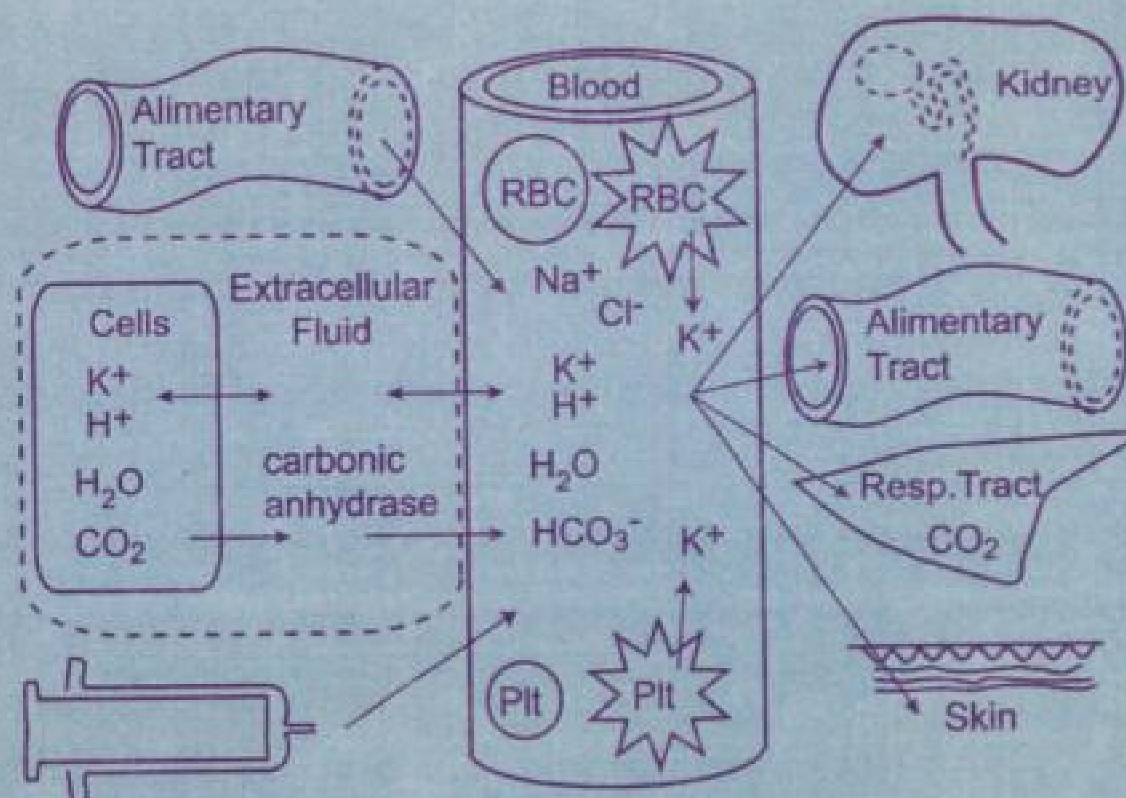
Fundamentals of

VETERINARY CLINICAL PATHOLOGY



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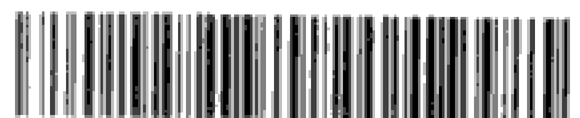

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Steven L. Stockham • Michael A. Scott

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Blackwell Publishing Professional
2121 State Avenue, Ames, Iowa 50014, USA

Orders: 1-800-862-6657
Office: 1-515-292-0140
Fax: 1-515-292-3348
Web site: www.blackwellprofessional.com

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First edition, 2002

Library of Congress Cataloging-in-Publication Data

Stockham, Steven L.
Fundamentals of veterinary clinical pathology / Steven L. Stockham and
Michael A. Scott.—1st ed.
p. ; cm.
Includes bibliographical references and index.
ISBN 0-8138-2561-X (alk. paper)
1. Veterinary clinical pathology.
[DNLM: 1. Animal Diseases—pathology. 2. Clinical Laboratory
Techniques—veterinary. 3. Pathology, Veterinary—methods. SF 772.6
S864f2002] 1. Scott, Michael A. (Michael Alan), 1957-II. Title.
SF772.6 .S76 2002
636.089'607—dc21

2002003002

The last digit is the print number: 9 8 7 6 5

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ACKNOWLEDGEMENTS

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, 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 or 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 long-time 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.

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PREFACE

The specialty of veterinary clinical pathology involves the sciences of basic pathology, hematology, clinical chemistry, cytology, and surgical pathology. The expertise of a veterinary clinical pathologist falls within two broad areas: (1) the generation of laboratory data, and (2) the interpretation of laboratory data. This textbook is focused on the latter, although analytical methods and concepts are provided so that the reader can better understand and interpret the results that are generated. Pertinent physiologic processes are also summarized so that pathologic alterations are better understood.

In most veterinary curricula, the educational objectives for the instruction in clinical pathology emphasize the interpretation of laboratory data. This textbook grew out of nearly 20 years of teaching a sophomore clinical pathology course that had these major goals for students:

- List or describe the clinical laboratory assays that are used to detect, define, or evaluate pathologic states in domestic animals.
- List or describe pathophysiologic syndromes, diseases, or other conditions that should be considered when results of clinical laboratory assays are abnormal.
- Explain the pathologic and non-pathologic (physiologic, procedural) processes that result in abnormal laboratory data.
- Identify via microscopy the cells, organisms, and other structures in clinical samples that indicate or suggest the presence of common pathologic states.

The title of this textbook, *Fundamentals of Veterinary Clinical Pathology,* was chosen because the book provides information to assist students and veterinarians in gaining a fundamental understanding of laboratory test results. In this context, *fundamental* should not be translated as *simple*, because fundamental understandings can require knowledge of many facts and their relationships. The prevailing theme throughout the book is explanation of the pathologic, physiologic, or analytical mechanisms responsible for abnormal laboratory data. Such information provides a fundamental understanding of laboratory data, diseases, and case management decisions. When the fundamental mechanisms are known and understood, the veterinarian is usually able to apply his or her knowledge to many species and disease variants. In this textbook, we limited the species content to the four major domestic mammals (dogs, cats, horses, and cattle), but most of the pathologic and non-pathologic processes occur in all mammals and in other animals.

The amount of information that must be understood to interpret laboratory data accurately can be overwhelming, especially for the sophomore veterinary student. When this textbook is used as an educational resource in a veterinary curriculum, course faculty are encouraged to use case-based discussions or clinical learning experiences so the facts and concepts can be learned in context and directed towards their application to the common disorders in veterinary medicine. With a basic understanding of pathologic processes and frequent practice applying facts and concepts, veterinarians can develop expertise in the interpretation of laboratory data.

This textbook concentrates on the laboratory data that relate to the disciplines of hematology and clinical chemistry. Chapters on the analysis of peritoneal, pleural, synovial, and cerebrospinal fluids, and on the microscopic examination of biopsy samples were excluded because several recent well-written and well-illustrated books already focus on these diagnostic procedures.

The extent to which one should reference facts and concepts in textbooks can be controversial; selecting the most appropriate reference from many possible ones can be challenging. We attempt-

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ed to cite references that document major facts and concepts or that provide additional information about certain processes, disorders, or diseases. The references we considered most appropriate varied with the topic and purpose of each citation, and included original articles, recent articles, review articles, and textbooks.

Many people choose academia for a career because of the opportunity to work in a mentally stimulating environment in which learning is a common goal. We discovered that the writing of this textbook was a great learning experience. We hope our efforts will facilitate learning by many others.

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Chapter 1

INTRODUCTORY CONCEPTS

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Table 1.1. Abbreviations and symbols in Chapter 1

$[fT_4]_{ed}$	free thyroxine concentration by equilibrium dialysis
[x]	concentration of x; x = analyte
CV	coefficient of variation
FN	false negative
FP	false positive
fT_4	free thyroxine
Hct	hematocrit
IFCC	International Federation of Clinical Chemistry
IUPAC	International Union of Pure and Applied Chemistry
PV(-)	predictive value of negative test
PV(+)	predictive value of positive test
ROC	receiver operating characteristic
sd	standard deviation
SI	Système International d'Unités
T_3	triiodothyronine
TN	true negative
TP	true positive
TRH	thyrotropin releasing hormone
tT_4	total thyroxine
U	international unit
URL	upper reference limit
USD	usual standard deviation
WRI	within reference interval

Note: See Table 1.3 for abbreviations of units of measurement and figure legends for abbreviations unique to figures.

CLINICAL PATHOLOGY

I. What is clinical pathology?

A. Definitions[1]

1. *Pathology* is the "branch of medicine that deals with the basis of disease, especially those structural and functional changes in organs and tissues causing or caused by a disease." In general terms, it is the study of disease.
2. *Clinical pathology* is a "subspecialty of pathology that deals with the use of laboratory methods (clinical chemistry, microbiology, hematology, . . .) for the diagnosis and treatment of disease." In general terms, it is the study of disease in the clinical environment using laboratory assays.

B. In veterinary medicine, clinical pathologists are trained as specialists in the disciplines of basic pathology, hematology (study of blood), clinical chemistry (study of physiologic and biochemical reactions), cytology (study of cells), and surgical pathology (study of disease via analysis of tissue samples obtained during surgery).

C. Veterinary clinical pathologists and other laboratory professionals (medical technologists, medical laboratory technicians, veterinary technicians) often work in a clinical laboratory that limits its assay "menu" (offered tests) to hematologic assays, clinical chemical assays, urinalysis, and clinical cytologic or histologic examinations. Other assays or diagnostic laboratory procedures are offered by specific laboratories (e.g., microbiology, histopathol-

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ogy, toxicology) that are supervised by microbiologists, histopathologists, or toxicologists, respectively.

II. Laboratory tests should be used with other diagnostic procedures. Before laboratory tests are used to pursue a possible diagnosis, it is imperative that two diagnostic procedures are done: (1) obtain a complete history, (2) perform a complete physical examination. With knowledge gained from these two basic procedures, a diagnostician can select diagnostic procedures to clarify or classify identified problems. Veterinarians frequently use the 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, cardiovascular) are relatively easily evaluated via visual or imaging methods (physical examination, radiography, ultrasonography), while other body systems (e.g., hemic, immune, urinary, endocrine) are better evaluated by laboratory tests.

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 (rule out) 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 therapy

MAJOR TYPES OF LABORATORY ASSAYS

I. Many laboratory tests or assays involve the analysis of body fluids (blood, serum, plasma, urine, peritoneal fluid, pleural fluid, cerebrospinal fluid, synovia), tissue samples, or feces. Most clinical laboratory procedures fall into one of three large groups (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 leukocyte concentration (count), erythrocyte concentration, platelet concentration
2. Semiquantitation of cell concentrations: calculated absolute leukocyte concentration, platelet estimate from blood film examination
3. Defining or classifying cells by microscopic features: toxic neutrophils, reactive lymphocytes, polychromatophilic erythrocytes, poikilocytes, microcytes, hypochromic erythrocytes, leukemic cells
4. Assessing the coagulation properties of blood: clotting times, platelet function assays
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, urea).
b. Semiquantitative analysis: Results are "within the ball park" (e.g., urine glucose, protein, and bilirubin concentrations by reagent pad chemistry assays).
c. Qualitative analysis: Results indicate that a substance is or is not present (e.g., fat present in pleural fluid).
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).

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b. Qualitative analysis: Results indicate that activity is or is not present (e.g., heme's peroxidase activity or leukocyte esterase 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., lymph node aspirates to diagnose lymphoma or histoplasmosis, aspirate of a skin tumor to determine if it is an inflammatory or neoplastic lesion, 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 in an attempt to define or classify abnormal tissue (i.e., inflammatory, neoplastic, toxic disorders) and perhaps establish an etiologic diagnosis.
3. Urine sediment analysis: Microscopic examination of urine to detect or semiquantify the presence of leukocytes, erythrocytes, casts, bacteria, crystals, or other structures.
4. Clinical parasitology: Microscopic analysis of fecal, urine, blood, or other sample to detect ova, oocytes, larvae, or other microscopic forms of parasites.

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.
A. Chapter 2 contains 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 through 18 have short sections that describe analytical principles that apply to individual analytes.

UNITS

I. SI units versus non-SI (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, 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 non-SI units.
B. In the context of units used for laboratory data, the basic units of measurement are shown in Table 1.2. 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 such a volume (1 m^3 = 1000 L) is rarely clinically relevant. Even the use of liter

Table 1.2. 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 (hr)
Volume	cubic meter (m^3)	liter (L)

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Table 1.3. Common units and abbreviations for laboratory values

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

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 of substances vary considerably, a veterinary medical professional should know the common abbreviations for the major units (Table 1.3).

D. Table 1.4 contains the formulas for the conversion of analyte concentrations from non-SI units to SI units; only analyte concentrations presented in this textbook are included. The table contains two types of formulas: (1) formulas that show the simple conversion factor that is used to calculate the numerical value of the SI unit,[2] and (2) formulas that show the conversion of the numerical value and concentration units. When available, the recommended smallest reportable increment of the SI unit is provided.[2] Similar information is contained in the analytical concept sections of each chapter.

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. The following examples illustrate the concepts.

A. A dog acutely lost a large amount of blood because of an injury. Because whole blood including erythrocytes was lost, the number of erythrocytes in the body is decreased. However, because plasma was lost with the erythrocytes, the erythrocyte concentration (number of erythrocytes per volume of blood) in the dog initially will not be decreased and thus the dog is not initially anemic. After fluid shifts restore plasma volume, the dog will have fewer erythrocytes in its body and a lower erythrocyte concentration in its blood.

B. You are told that a cat's serum sodium was increased. Does this mean the cat has more sodium in its body? Well, it might. However, the increased serum sodium concentration 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 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 maybe 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 it might not. A percentage is always relative; the same number in the numerator and a smaller number in the denominator will result in an increased percentage.

E. You are told that the myeloid: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,

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Table 1.4. Formulas for conversion of non-SI units to SI units of concentration

Analyte[a]	Conversion factor formulas[b]	Complete conversion formulas	Increment[c]
ACTH	pg/mL × 0.2202 = pmol/L	pg/mL ÷ 4541 pg/pmol × 1000 mL/L = nmol/L	1 pmol/L
Albumin	g/dL × 10 = g/L	g/dL × 10 dL/L = g/L	1 g/L
Aldosterone	ng/dL × 27.74 = pmol/L	µg/dL ÷ 360.5 µg/µmol × 1000 nmol/µmol × 10 dL/L = nmol/L	10 pmol/L
Bile acid (total)	mg/mL × 2.547 = mmol/L	mg/dL ÷ 392.6 mg/mmol × 1000 mL/L = mmol/L	0.2 mmol/L
Bt	mg/dL × 17.10 = µmol/L	mg/dL ÷ 584.8 mg/mmol × 1000 µmol/mmol × 10 dL/L = µmol/L	2 µmol/L
Cholesterol	mg/dL × 0.02586 = mmol/L	mg/dL ÷ 386.7 mg/mmol × 10 dL/L = mmol/L	0.05 mmol/L
Cl^-	mEq/L × 1 = mmol/L	mEq/L × 1 mmol/mEq = mmol/L	1 mmol/L
Cl^-	mg/dL ÷ 3.55 = mmol/L	mg/dL ÷ 35.5 mg/mmol × 10 dL/L = mmol/L	1 mmol/L
Cortisol	µg/dL × 27.59 = nmol/L	µg/dL ÷ 362.45 µg/µmol × 1000 nmol/µmol × 10 dL/L = nmol/L	10 nmol/L
Creatinine	mg/dL × 88.4 = µmol/L	mg/dL ÷ 113.1 mg/mmol × 1000 µmol/mmol × 10 dL/L = mmol/L	10 µmol/L
Cyanocobalamin	pg/mL × 0.7378 = pmol/L	pg/mL ÷ 1355 pg/pmol × 1000 mL/L = pmol/L	10 pmol/L
fCa^{2+}	mg/dL × 0.2495 = mmol/L	mg/dL ÷ 40.08 mg/mmol × 10 dL/L = mmol/L	0.01 mmol/L
fCa^{2+}	mEq/L × 0.5 = mmol/L	mEq/L × 0.5 mmol/mEq = mmol/L	0.01 mmol/L
Fe	µg/dL × 0.1791 = µmol/L	µg/dL × 0.01791 µmol/µg × 10 dL/L = µmol/L	1 µmol/L
Ferritin	ng/mL × 1 = µg/L	ng/mL × 1 µg/1000 ng × 1000 mL/L = µg/L	10 µg/L
Fibrinogen	mg/dL × 0.01 = g/L	mg/dL × g/1000 mg × 10 dL/L = g/L	0.1 g/L
Folate	ng/mL × 2.266 = nmol/L	ng/mL ÷ 441.3 ng/nmol × 1000 mL/L = nmol/L	2 nmol/L
fT_4	ng/dL × 12.87 = pmol/L	ng/dL ÷ 777 ng/nmol × 1000 pmol/nmol × 10 dL/L = pmol/L	1 pmol/L
Globulin	g/dL × 10 = g/L	g/dL × 10 dL/L = g/L	1 g/L
Glucose	mg/dL × 0.05551 = mmol/L	mg/dL ÷ 180.1 mg/mmol × 10 dL/L = mmol/L	0.1 mmol/L
HCO_3^-	mEq/L × 1 = mmol/L	mEq/L × 1 mmol/mEq = mmol/L	1 mmol/L
Hgb	g/dL × 10 = g/L	g/dL × 10 dL/L = g/L	1 g/L
IRG	pg/mL = ng/L	pg/mL ÷ 1000 pg/ng × 1000 mL/L = ng/L	10 ng/L
IRI	µU/mL × 7.175 = pmol/L	µU/mL ÷ 139.4 µU/pmol × 1000 mL/L = pmol/L	5 pmol/L
IRI	µg/L × 172.2 = pmol/L	µg/L ÷ 5.807 µg/µmol × 1000 pmol/µmol = pmol/L	5 pmol/L
K^+	mEq/L × 1 = mmol/L	mEq/L × 1 mmol/mEq = mmol/L	0.1 mmol/L
K^+	mg/dL ÷ 3.9 = mmol/L	mg/dL ÷ 39 mg/mmol × 10 dL/L = mmol/L	0.1 mmol/L
MCHC	g/dL × 10 = g/L	g/dL × 10 dL/L = g/L	—
Na^+	mEq/L × 1 = mmol/L	mEq/L × 1 mmol/mEq = mmol/L	1 mmol/L
Na^+	mg/dL ÷ 2.3 = mmol/L	mg/dL ÷ 23 mg/mmol × 10 dL/L = mmol/L	1 mmol/L

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NH_3	μg/dL × 0.5871 = μmol/L	μg/dL ÷ 17.03 μg/μmol × 10 dL/L = μmol/L	5 μmol/L
NH_4^+	μg/dL × 0.5543 = μmol/L	μg/dL ÷ 18.04 μg/μmol × 10 dL/L = μmol/L	5 μmol/L
Pi	mg/dL × 0.3229 = mmol/L	mg/dL ÷ 30.97 mg/mmol × 10 dL/L = mmol/L	0.05 mmol/L
Platelet	(# × 10^3)/μL) × 10^6 = # × 10^9/L	(# × 10^3)/μL) × 10^6 × (# × 10^3)/μL × 10^6 μL/L = # × 10^9/L	10 × 10^9/L
RBC	(# × 10^6/μL) × 10^6 = # × 10^{12}/L	(# × 10^6/μL) × 10^6 μL/L = # × 10^{12}/L	—
T_3	ng/dL × 0.01536 = nmol/L	ng/dL ÷ 651 ng/nmol × 10 dL/L = nmol/L	0.1 nmol/L
T_3	pg/dL × 15.36 = nmol/L	pg/dL ÷ 651 pg/pmol × 1000 pmol/nmol × 10 dL/L = nmol/L	0.1 nmol/L
tCa^{2+}	mg/dL × 0.2495 = mmol/L	mg/dL ÷ 40.08 mg/mmol × 10 dL/L = mmol/L	0.02 mmol/L
tCa^{2+}	mEq/L × 0.5 = mmol/L	mEq/L × 0.5 mmol/mEq = mmol/L	0.02 mmol/L
TIBC	μg/dL × 0.1791 = μmol/L	μg/dL × 0.01791 μmol/μg × 10 dL/L = μmol/L	1 μmol/L
tMg^{2+}	mg/dL × 0.4114 = mmol/L	mg/dL ÷ 24.31 mg/mmol × 10 dL/L = mmol/L	0.02 mmol/L
tMg^{2+}	mEq/L × 0.5 = mmol/L	mEq/L × 0.5 mmol/mEq = mmol/L	0.02 mmol/L
Total protein	g/dL × 10 = g/L	g/dL × 10 dL/L = g/L	1 g/L
Triglyceride	mg/dL × 0.01129 = mmol/L	mg/dL ÷ 885.7 mg/mmol × 10 dL/L = mmol/L	0.05 mmol/L
TSH	μg/dL × 10 = ng/mL	μg/dL × 1000 ng/μg ÷ 100 mL/dL = ng/mL	—
tT_4	μg/dL × 12.87 = nmol/L	μg/dL ÷ 777 μg/μmol × 1000 nmol/μmol × 10 dL/L = nmol/L	1 nmol/L
tT_4	ng/mL × 1.287 = nmol/L	ng/mL ÷ 777 ng/nmol × 1000 mL/L = nmol/L	1 nmol/L
UN	mg/dL of UN × 0.3570 = mmol/L of urea	mg/dL ÷ 28.01 mg/mmol × 10 dL/L = mmol/L of urea	0.5 mmol/L
Urea	mg/dL of urea × 0.1665 = mmol/L of urea	mg/dL ÷ 60.06 mg/mmol × 10 dL/L = mmol/L of urea	0.5 mmol/L
WBC	#/μL × 10^6 = # × 10^6/L	#/μL × 10^6 μL/L = # × 10^6/L	—
Xylose	mg/dL × 0.06661 = mmol/L	mg/dL ÷ 150.1 mg/mmol × 10 dL/L = mmol/L	0.1 mmol/L

[a]ACTH = adrenocorticotrophic hormone, Bt = total bilirubin, Cl^- = chloride ion, fCa^{2+} = free calcium ion, Fe = iron, fT_4 = free thyroxine, HCO_3^- = bicarbonate ion, Hgb = hemoglobin, IRG = immunoreactive glucagon, IRI = immunoreactive insulin, K^+ = potassium ion, MCHC = mean cell hemoglobin concentration, Na^+ = sodium ion, NH_3 = ammonia, NH_4^+ = ammonium ion, Pi = inorganic phosphorus, RBC = red blood cell, erythrocyte, SI = Système International d'Unités, T_3 = triiodothyronine, tCa^{2+} = total calcium ion, TIBC = total iron binding capacity, tMg^{2+} = total magnesium ion, TSH = thyroid stimulating hormone, tT_4 = total thyroxine, UN = urea nitrogen, WBC = white blood cell, leukocyte.

[b]*Source:* Lundberg GD, Iverson C, Radulescu G. 1986. Now read this: The SI units are here. J Am Med Assoc 255:2329-2339.

[c]Recommended smallest reportable increment of the SI unit.

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or both? Or is the ratio increased because 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 concentration. Because the concentrations of all substances in urine are dependent on the conservation of water by the kidneys, the increased protein concentration could result from increased water conservation and not increased protein loss via the urinary system.

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 the results we expect to find in healthy animals. These intervals are used to help detect pathologic states. Other terms that are used as synonyms include *normals, normal values, normal range,* and *reference range.*
   B. In an attempt to establish uniform usage of terms, the following terms and definitions have been recommended by an Expert Panel of the International Federation of Clinical Chemistry.[3]
      1. *Reference individual:* an animal selected by using defined criteria
      2. *Reference population:* consists 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:* 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:* distribution of reference values, which is not necessarily Gaussian (bell-shaped curve)
      6. *Reference limit:* the upper or lower values of the reference interval as derived from a reference distribution
      7. *Reference interval:* an interval between and including the two reference limits
      8. *Observed value:* a value obtained by observation or measurement that is to be compared to the reference interval
   C. Use of the term *reference range* is discouraged for two reasons.
      1. Statistically, a range is the difference between highest and lowest observations; e.g., the range is 40 if the highest observation was 50 and the lowest was 10.
      2. Some consider the *range of values* to include all measured values from the lowest to the highest; a reference interval does not include all reference values.
   D. Using the terms *normal* and *abnormal* to describe laboratory test results can be misleading and is discouraged.
      1. A laboratory result can be WRI but still reflect a pathologic process. For example, a serum sodium concentration that is WRI in a dehydrated animal indicates that the animal has lost both water and sodium from its body.
      2. Sick animals usually will have some laboratory results that are WRI. Conversely, some apparently healthy animals will have laboratory results outside of the reference interval. Because the laboratory test results for certain diseases overlap between "sick" and "healthy" animals, it is inappropriate to classify a patient as "normal" based just on test results.

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3. It is difficult to define "normal" because many variations that may appear to be "abnormal" are due to physiologic, dietary, environmental, or other nonpathologic factors.

II. Establishment of reference intervals: A complete description of the process of establishing reference intervals is beyond the scope of this book and the reader is referred to publications.[4,5] The major steps in the process are as follows:

A. Select criteria for reference individuals. Criteria could include species, age, and method of determining health status.
   1. Initially, this may seem like a simple task because the criteria define clinically healthy adult animals. Generally, we want to sample a broad group of animals so that the reference interval is useful for a broad group of patients. However, it can become more complex when there are potentially clinically relevant differences that can be seen between breeds (e.g., some Akita dogs have smaller erythrocytes), physiologic variations (e.g., milking versus non-milking cattle; high altitude versus sea level), or nutritional variations.
   2. Even if a laboratory is successful in establishing reference intervals for adult animals, how about appropriate reference intervals for neonates, nursing animals, or weanlings? Critical assessment of patient values requires that criterion-matched reference intervals be established.

B. Establish a reference sample group. It is preferable to have at least 60 animals that meet selected criteria.
   1. Authorities state that at least 120 individuals are needed;[5] obtaining such numbers is often not accomplished in veterinary medicine. A more realistic number of 60 qualified individuals may be sufficient if a Gaussian distribution is present;[4] attempts to establish reference intervals with fewer individuals frequently result in weak intervals that are often questioned by clinical observations.
   2. Obtaining quality samples from 60 qualified individuals is the most difficult aspect of establishing reference intervals. Typically, samples are collected from animals that are seen because of yearly vaccinations or for elective surgical procedures. Collecting many samples from one kennel, one cattery, one stable, or one herd is typically not recommended because the animals may lack the breed and other physiologic variations needed for representative reference intervals. For example, reference intervals established for 60 beagles may not be appropriate for healthy dogs of other breeds, but they would be great reference intervals for beagles. Similarly, reference intervals established for cows from one dairy may not be representative of healthy cattle in other dairies or in beef herds.

C. Measure or determine the reference values. Analyze the sample for the substance of interest (analyte).
   1. This is the most expensive aspect of establishing reference intervals. For example, glucose tests of 60 sera at $5 each cost $300. Since we might measure 20 analytes in sera, with each costing the same amount, $6000 would be spent—and this is for only one species. If the analyses were completed for each major species (bovine, canine, equine, feline), $24,000 would be spent for the routine chemical assays. A similar amount might be spent for hematologic assays and special assays.
   2. This expense is magnified when a laboratory changes laboratory equipment and must establish new reference intervals for a new instrument every 5 to 7 years (expected life span of most instruments).

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D. Determine the reference distribution. Apply statistical methods to determine whether data have a Gaussian or a skewed distribution.
   1. Many analyte concentrations will fit a normal (Gaussian) distribution, especially those analyte concentrations that are tightly regulated by physiologic systems (e.g., glucose, sodium, potassium, and free calcium concentrations).
   2. Many analyte concentrations (e.g., serum enzyme activities) will not fit a Gaussian distribution; their data may have positive or negative skewness.
E. Determine reference limits and reference intervals. Use methods to remove outliers and then select the central 95% of the reference values.
   1. Limits may be defined by a stated fraction of reference values that are less than or equal to a certain result. For example, 2.5% of the values are > 150 and 2.5% of values are < 50 and thus the reference limits are 50 and 150. The reference interval would be 50–150.
   2. When data have been shown to fit a Gaussian distribution, then parametric methods can be used to establish reference limits. The reference limits are the calculated values that represent the mean ± 2 sd.
   3. When the distribution is not Gaussian, the data should either be transformed into a Gaussian distribution or, more commonly, the data within the top and bottom 2.5 percentiles are removed by nonparametric methods.
   4. Fig. 1.1 shows the differences between Gaussian and skewed distributions and the reference intervals obtained from such distributions.
   5. It is important to understand that reference intervals represent results expected in 95% of the healthy animals (i.e., in 19 of 20 healthy animals).

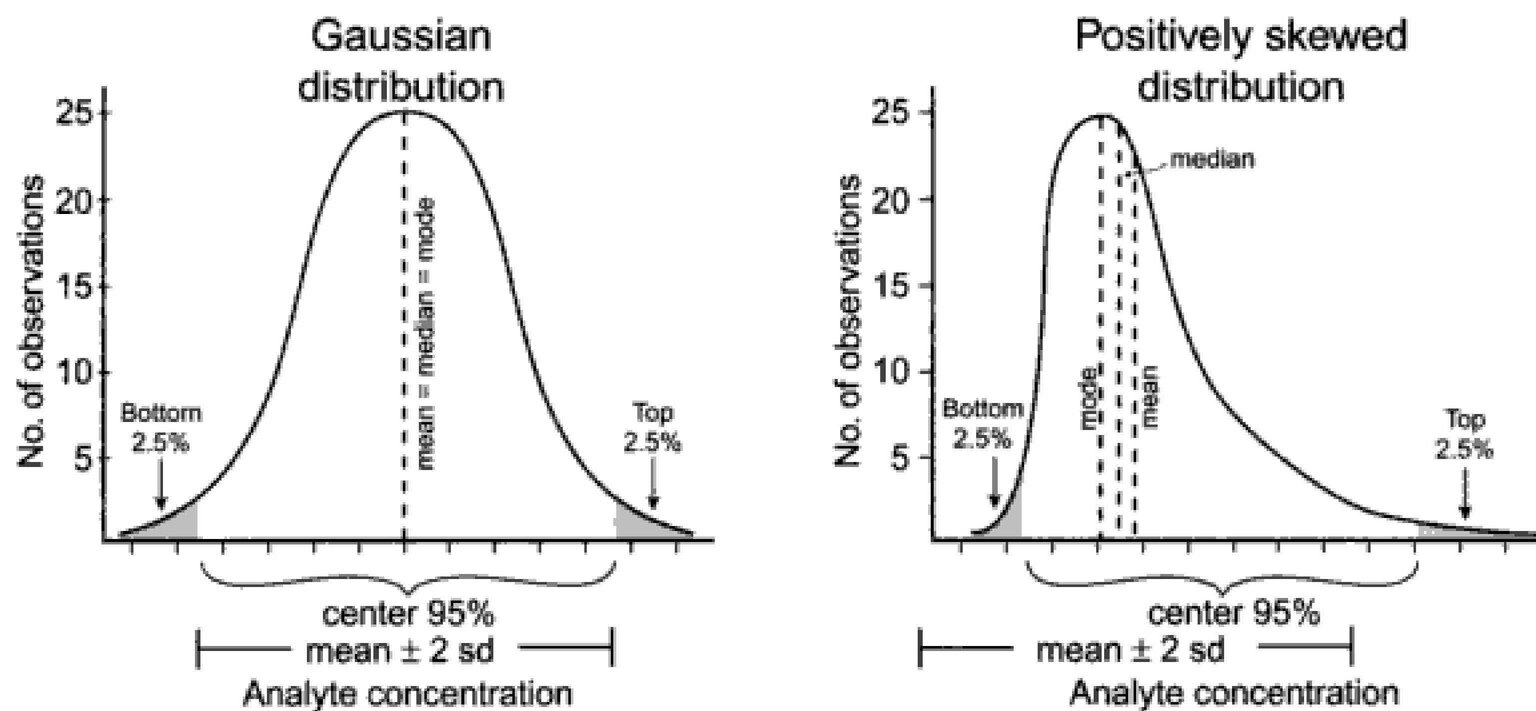

Fig. 1.1. Reference distribution.

In the *left graph,* the reference values conformed to a Gaussian distribution. If data have this distribution, then mean ± 2 sd will represent the central 95% of the reference values and thus the reference interval. If values have a Gaussian distribution, the mean, median, and mode values will be 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 lower reference limit would be below the lowest reference value and the upper reference limit 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 non-Gaussian distribution.

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a. Therefore, 1 of 20 healthy animals is expected to have a measured value outside of the reference interval. The value that is outside the reference interval but still represents a value from a healthy animal is expected to be close to the reference limits. A marked difference from the reference interval probably represents a pathologic state.
b. When examining multiple test results for one animal, then the concepts of multivariate comparison apply.[6] Because there is a 95% (or 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 is $1 - 0.95^n$. Thus, for a panel of 20 test results, there is a 64% $[100 \times (1 - 0.95^{20})]$ chance that at least one value is outside the reference interval. With a panel of five test results, there is a 23% chance. However, that value should be close to the lower or upper reference limit. This concept is important when laboratory tests are requested for clinically healthy animals for a "yearly health profile" or a "geriatric profile."

QUALITY OF LABORATORY RESULTS

I. Major determinants: Laboratory test results will be of the most benefit if they are consistently correct, i.e., from patient to patient, day to day, month to month. Three major factors determine whether results are valid: (1) quality of sample, (2) quality of analysis, (3) quality of laboratory and patient records. When results are not what a veterinarian expects, he or she might say, "I do not believe those results; there must have been a lab error." When such conclusions are formed, it is important to remember that there are many potential reasons for an erroneous laboratory test result.
  A. Sample quality: A laboratory test result can only be as good as the sample.
    1. Sample collection
      a. Properly prepared patient (e.g., the animal should be fasted for at least 8 hr prior to collection of most blood samples).
      b. Proper collection technique (e.g., clean venipuncture to minimize trauma to blood cells [prevent hemolysis] and to not activate clotting proteins or platelets).
      c. Proper collection container (e.g., sterile versus nonsterile or clot tube versus an EDTA tube).
      d. Proper anticoagulants when needed (e.g., EDTA versus heparin, sodium heparin versus lithium heparin).
      e. Adequate volume for assays (e.g., to obtain 1 mL of serum, at least 3 mL of blood are usually needed).
    2. Sample handling
      a. Proper labeling of all specimens (e.g., animal identification [name or number], date, and time of collection).
      b. Samples kept at appropriate temperature prior to and after processing, during shipment, or during storage (e.g., 25°, 4°, or -25°C).
      c. Prompt processing (e.g., for a labile analyte, process immediately so analyte does not deteriorate).
  B. Quality analysis of sample
    1. Method appropriate for species (e.g., an instrument designed to measure the relatively large human erythrocytes may not provide accurate measurement of smaller erythrocytes from the domestic mammals).

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2. Quality of instruments and equipment (i.e., generally "you get what you pay for," but a quality instrument remains a quality instrument only if it is properly maintained).
3. Quality of reagents (e.g., fresh and within expiration date, being used according to instructions).
4. Quality of laboratory technique (e.g., person-to-person variation, training of person, inherent procedural difficulty).
5. Quality control program (quality assurance program) (e.g., the laboratory personnel subscribe to and adhere to internal assessment procedures to assure that all parts of the quality analysis of the sample are maintained daily).

C. Recording and reporting results
1. Transcriptional errors minimized (e.g., errors can be made during manual transcription of results to a laboratory report or during keyboard entering of data into computers).
2. Timely results (i.e., quality results are valuable only when they can be used by the requesting veterinarian).

II. Analytical properties of assays: From the analytical perspective, the best clinical laboratory assay is one that consistently measures the true concentration of the substance and at concentrations that are clinically relevant. When evaluating and comparing laboratory assays, there are five properties that can be assessed.

A. *Analytical precision:*[7] the ability of an assay to get the same result if a sample is analyzed several times; also called *reproducibility* or *random analytical error* (Fig. 1.2*a*)
1. The need for analytical precision is dependent on the degree of variation that can be accepted as a random variation (error). When management of a case requires that small changes in concentration reflect a biologic change and not analytical variation, then an assay needs to have high precision. However, if a wide range of measured values can be accepted, then high precision is not needed.
2. Control solutions are used to assess assay performance. Repeated analysis of control solutions allows assessment of precision.
   a. A control solution contains an analyte's concentration that can be a known specific concentration (like a standard solution), but more frequently the concentration is determined by multiple measurements by the same assay for which precision is being assessed.
   b. If results for a control solution are within acceptable limits, then the assay was probably performed correctly and thus the patient's result is probably valid. Control solution results that are outside acceptable limits suggest instrument malfunction, deteriorated reagents, or poor analytical techniques. The patient's result determined by the same assay may or may not be accurate.
3. Precision is frequently expressed in clinical laboratories by the method's CV (Eq. 1.1).

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

   a. A method's CV is determined from replicate analysis within an assay run and between different runs of the same assay.
      (1) A within-assay CV 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

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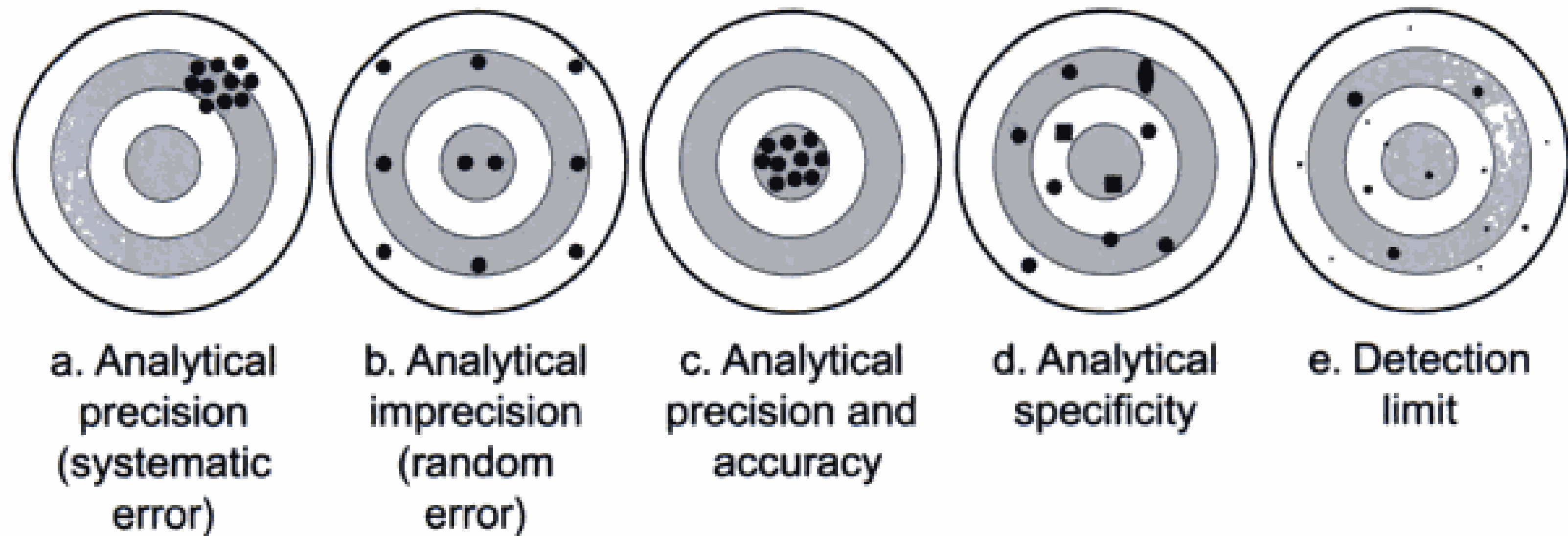

Fig. 1.2. Illustrations of analytical properties of assays.

- *Analytical precision (systematic error)* (Fig. 1.2*a*): Because the 10 holes in the target are tightly clustered, the target shooter was precise even though consistently inaccurate.
- *Analytical imprecision (random error)* (Fig. 1.2*b*): 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.
- *Analytical precision and accuracy* (Fig. 1.2*c*): Because all 10 holes in the target are tightly clustered in the middle of the target, the shooter was accurate and precise.
- *Analytical specificity* (Fig. 1.2*d*): 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.
- *Detection limit and analytical sensitivity* (Fig. 1.2*e*): 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 size that your eyes can reliably differentiate. If your eyes can reliably detect the different sizes of all 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 bullet whose diameter was 75% of the previous shot.

poor within-assay CV, the sample's analyte concentration is determined by analyzing the sample in duplicate or triplicate and then calculating a mean concentration.

(2) A between-assay CV represents the random error within one run of the assay plus the error from additional runs of the assay using the same sample. The within-assay CV will be smaller than the between-assay CV.

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

(1) An assay's CV will vary 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 tend to be higher at the lower analyte concentrations.

(2) If critical clinical decisions are made when there are minimal changes in an analyte's concentration, 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.

(3) Because CV values are expressed as percentages, they have different meanings at different concentrations.

(a) If an assay has a CV of 10% at all concentrations, then the assay's sd would be 0.1 at an analyte concentration of 1, 1 at an analyte concentra-

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tion of 10, 10 at an analyte concentration of 100, etc. Depending on the analyte and the amount of biologic variation, a CV of 10% can be completely unacceptable analytical variation or be very acceptable.

(b) If the assay's sd is 10, its CV values would be 50% at a mean concentration of 20, 10% at a mean of 100, 1% at a mean of 1000, etc.

(4) As a rule of thumb, a change in concentration in patient samples that is greater than 3 times the USD is due to biologic variation; a change that is less than 2 times the USD may be only analytical variation. USD is an average of sd values from 3 to 6 consecutive months of quality control values. The "3 times USD" is an estimate of significant change limit.[8]

(a) If the USD for sodium concentration at 150 mmol/L is 1.5 mmol/L and a patient's sodium concentration 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 < 2 times the USD. However, a value of 145 mmol/L on day 2 (a change > 3 times USD) probably represents a true biologic change.

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

(5) The CV percentages and USD values may vary considerably between assay methods and the percentages or values are frequently not known by the diagnostician. However, such information can improve the interpretation of laboratory data because changes can be recognized as potential analytical variation or confidently classified as biologic change.

B. *Analytical accuracy* (IFCC definition):[7] the closeness of the agreement between the measured value of an analyte and its "true" value (Fig. 1.2*b*)

1. The methods of establishing the "true" values vary considerably. At times, the true value is established by a reference method (e.g., method developed by the National Institute of Standards and Technology). If such standards have not been developed, the "true" value might represent the mean concentration determined by numerous observations by 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 (Fig. 1.2*c*).

3. 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 determined by a reference method.

b. Reference standard solutions are not the same as calibration standard solutions. *Calibration standard solutions* are commercially prepared, are used to calibrate an instrument or method, and should closely agree with reference standard solutions.

C. *Analytical specificity:*[7] the ability of an assay to detect only the substance of interest (analyte) or freedom from interfering substances (Fig. 1.2*d*)

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1. Analytical specificity is related to analytical accuracy because an assay cannot be accurate if there is a nonspecific reaction 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 if other hexoses might be present in the sample.
3. Substances may interfere with an assay in many ways.
   a. The substance may be very similar chemically and the assay may react with either the substance of interest or the interfering substance.
   b. The substance may produce the same substance or response that is detected in the assay system.
      (1) The presence of glucose may be detected when a chemical reaction produces H_2O_2. A substance may 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 may interfere with light transmission through a sample and thus the results of the assay are changed by artifactual spectral changes and not by a change resulting from a chemical reaction.
4. Depending on how a substance interferes with an assay, it may lead to either falsely increased or falsely decreased concentrations—positive interference or negative interference, respectively.

D. *Detection limit* (IUPAC definition):[7] the smallest concentration or quantity of an analyte that can be detected with reasonable certainty for a given analytical range (Fig. 1.2*e*)
   1. An assay's detection limit involves the ability of the assay to differentiate background "noise" from a true change due to the presence of an analyte.
   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.

E. *Analytical sensitivity* (IUPAC definition):[7] slope of the calibration curve and the ability of an analytical procedure to produce a change in the signal for a defined change of the quantity
   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 (or 5000 mg/dL) from 5.1 g/dL (5100 mg/dL).
   2. Analytical sensitivity should not be confused with detection limit. The two are related because both relate to small changes in concentration. However, *analytical sensitivity* applies to changes within an assay's analytical range, whereas *detection limit* applies to the lowest limit of the analytical range.

DIFFERENCES IN LABORATORY METHODS AND THEIR RESULTS

I. Not only do veterinarians face challenges of species variations, they also must deal with the different results generated by different laboratories and different laboratory methods. There are many potential reasons for the differences; some of the differences are due to random

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errors that are not preventable, others are due to analytical or systematic errors (bias), and others are because of poor analytical methods that produce inaccurate results.

II. Examples of differences
   A. Fig. 1.3 illustrates results of a survey completed by the Veterinary Laboratory Association (VLA) as part of a quality assurance program. For the survey, the VLA distributed aliquots of a pooled serum to laboratories that subscribed to its quality assurance program. After analysis of the distributed sample, each subscribing laboratory sent its results to the association for compilation and analysis. The association then distributed the results of the analyses.

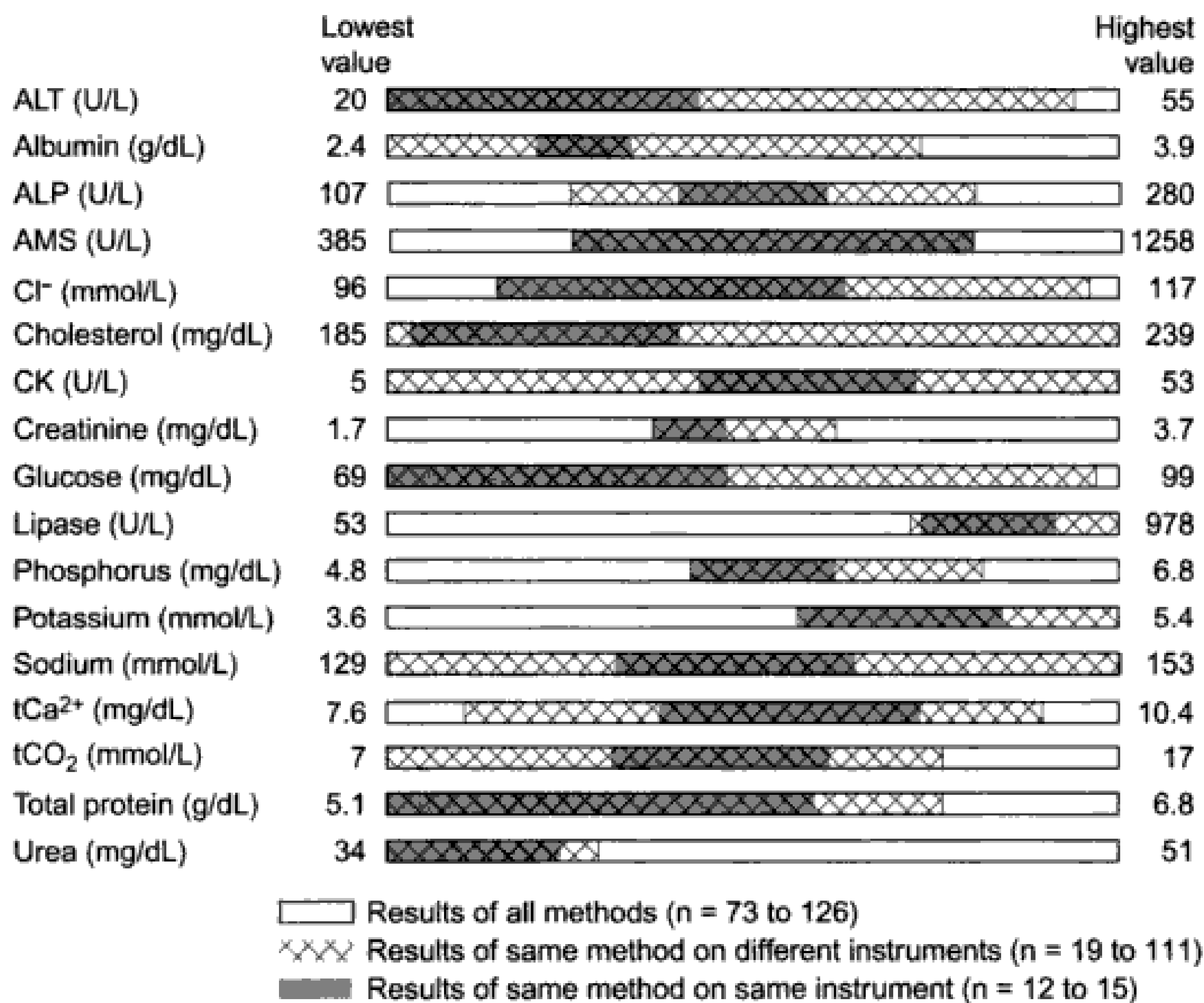

Fig. 1.3. Results from similar and different assay methods that analyzed aliquots of one canine serum as part of one survey completed by the VLA during 2000. (ALT = alanine transaminase, ALP = alkaline phosphatase, AMS = amylase, Cl^- = chloride, CK = creatine kinase, tCa^{2+} = total calcium, tCO_2 = total carbon dioxide)

Ranges for most measured analyte concentrations or enzyme activities were extracted from graphs within the survey's report, but data for ALP, AMS, and LPS activities were obtained from VLA because graphed bars were too small to be reliably interpreted.

- For nearly every measured analyte, 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.
- The greatest differences occur when different assay methods are used, but frequently there were large differences when the same assay method was used on different machines (e.g., ALT, ALP, AMS, cholesterol, CK, glucose, sodium, and tCa^{2+}).
- For the data used in this figure, the results of the same method on same instrument represent data for the same dry chemistry reagent system on the same type of instrument. Even then, the lack of agreement emphasizes the need for the establishment of reference intervals by each laboratory.

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B. Careful review of the data in Fig. 1.3 can be disturbing because there are marked differences in the results generated by laboratories. It is beyond the scope of this textbook to describe the possible reasons for the differences in detail. In general, the results may vary because of one or more of the following:
   1. Commercial standard solutions may be poorly calibrated. Although there are strict requirements for development of human assays, there are not the same requirements for veterinary assays.
   2. For assays that measure enzyme activity or depend on enzymatic activity within the assay, there can be marked differences in enzyme activity because of different substrates (reagents), reaction temperatures, or pH of assay systems.
   3. Even though two laboratories might have the same chemistry instrument and assay method, the laboratories might purchase reagents from different companies. The reagents might have the same constituents but at different concentrations.

C. Because of the potential marked differences in results determined by laboratories, several recommendations are made.
   1. Sample collection, processing, and transport should follow policies that minimize the chances of sample damage.
   2. Samples should be submitted to *veterinary* laboratories that adhere to stringent quality assurance guidelines.
   3. Your patient's results should be compared against appropriate reference intervals established for the same assay method in the same laboratory.
      a. Reference intervals established for another assay can be very misleading. Comparison against reference intervals published in textbooks is discouraged.
      b. Reference intervals provided by the manufacturer of an instrument should be carefully scrutinized. The manufacturer should provide details about the reference intervals, including criteria used to select reference individuals, number of animals in reference sample group, and method of determining the reference limits. If the manufacturer's reference intervals are going to be used, then split samples should be analyzed by the manufacturer and your laboratory to ascertain if there is excellent agreement in the assays' results.
   4. A good diagnostician detects and confirms abnormalities. If a laboratory result is not consistent with your impression of the case, you should ask the laboratory to repeat the assay or you should submit another sample to confirm a significant abnormality.
   5. You should establish a strong professional relationship with your laboratory's personnel. The quality laboratory will strive to provide you quality and timely results. You should strive to provide the laboratory with quality samples.

WHICH LABORATORY DO YOU USE?

I. Many factors must be considered before you decide how you will obtain laboratory data for your patients. Basically, there are three major options: in-house laboratory, veterinary reference laboratory, and 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?
   C. How much will it cost to analyze the sample?
   D. How important is a short turnaround time?

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II. In-house laboratory (in a veterinary clinic or hospital)
   A. Advantages
      1. May allow 24-hour access to laboratory data
      2. Shorter turnaround times, which are sometimes required for emergency cases
      3. Fresh samples and thus fewer concerns about sample deterioration
   B. Disadvantages
      1. Capital expenditure of several thousand dollars required and equipment depreciates rapidly
      2. Need to maintain inventory of reagents and supplies
      3. Need personnel trained to operate and maintain equipment
      4. Need to follow a quality assurance program; you may be legally responsible for documenting quality of laboratory data
      5. Typically need high sample volume to generate enough income to cover expenses
      6. Reference intervals typically are provided by manufacturer of instruments or assay and may or may not be quality reference intervals.
      7. May need to ship a malfunctioning instrument to manufacturer for repairs and thus need a replacement instrument or backup methods

III. Veterinary reference laboratory
   A. Advantages
      1. Laboratory personnel are typically trained to provide quality analysis of veterinary samples.
      2. Diagnostic support may be available from veterinary clinical pathologists in the laboratory.
      3. Reference intervals should be appropriate for species.
      4. Many more diagnostic assays are typically available than are available in an in-house laboratory.
      5. Cost for sample analysis is more clearly determined and thus can be charged to the client on a sample-by-sample basis.
   B. Disadvantages
      1. Not all analytes are stable and thus some deteriorate during shipment; some analytes require special shipping.
      2. Turnaround times vary with location: some are available the same day and most are available the next day except for special tests. Some laboratories offer courier service and results reported via fax machines or e-mail.

IV. Laboratory in local human hospital
   A. Advantage: Turnaround times can be within hours.
   B. Disadvantages
      1. Quality reference intervals for veterinary samples may not be established by the laboratory.
      2. Assay methods may not be appropriate for veterinary samples.
      3. Technicians and technologists may not be trained to correctly identify species variations or diseases that are unique to veterinary samples.
      4. Human pathologists frequently are very interested in helping but lack the training in the diseases of domestic animals and variations seen in veterinary samples.
      5. The fees charged by laboratories may be relatively high.

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EVALUATING AND VALIDATING LABORATORY METHODS[9]

I. Reasons for evaluating laboratory methods
   A. New or revised laboratory methods are evaluated for clinical use because of recent research findings, because newer assays might be more accurate, more precise, less expensive, or less difficult, or because an instrument was purchased to replace an outdated or malfunctioning instrument.
   B. A clinical perspective needs to be considered for new or revised laboratory methods. 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 the sources of analytical error?
   A. Each assay system has it own inherent *random error* (see CV [coefficient of variation] in Quality of Laboratory Results, II, earlier in this chapter). Typically, the more precise the assay, the better it is. But sometimes, the most precise methods are too expensive or time consuming.
   B. Besides random error, a method may not provide accurate results because of *systematic error* (bias). For example, the mean concentration of a new method might be consistently 5 mmol/L too high compared to the mean value determined by a reference method.
   C. *Accuracy* is a relative term, and an assay's analytical accuracy is sometimes difficult to assess in a clinical laboratory. An assay's result may be compared against one of two "true" values:
      1. The mean concentration determined by the reference method (a gold standard)
      2. The mean concentration determined by numerous analyses by comparative methods (all laboratories using the same instrument and reagents)
   D. Acceptable analytical performance
      1. The most accurate, precise, specific, or sensitive assay typically is not needed in a clinical environment. However, the assay does need to meet requirements for a clinically useful assay.
      2. Criteria for acceptable performance of clinical assays have been proposed and they vary considerably between analytes. A hematocrit method might be considered acceptable if it provides results within 6% of the target value (e.g., 37.6%–42.4% for a Hct of 40%). Or a cortisol method might be considered acceptable if cortisol concentrations are within 25% of target values.[7,9]

III. Validation methods: After available information has led to a conclusion that a new or revised assay should be evaluated, three stages of validation have been recommended.
   A. Familiarization: This stage includes establishment of a working procedure, initial assessment of the analytical range, and calibration.
   B. Preliminary validation: In this stage, several studies are completed, including within-run replication, interference studies, and recovery studies.
   C. Detailed validation: If initial results are satisfactory, then the final validation includes replication studies, comparison of methods, statistical analyses, determination of acceptable performance criteria, and establishment of reference intervals.

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IV. Implementation phase
   A. If results of the validation procedures indicate that the assay will be used in the laboratory, then it needs to be incorporated into the daily routine of the laboratory, including maintenance of equipment, reagent inventory, and a quality assurance program.
   B. When the assay is finally ready for clinical use, clinicians are notified of its availability, expected precision at major decision limits, and characteristics of reference intervals.

DIAGNOSTIC PROPERTIES AND PREDICTIVE VALUE OF LABORATORY ASSAYS

I. As mentioned earlier, a frequent purpose of analyzing a patient's sample is to detect or confirm the presence of a disease state. But if a laboratory test result is outside the reference interval, how likely is it that the patient has a certain disorder? Similarly, if a laboratory test result is WRI, how certain are we that the animal doesn't have a certain disease or pathologic state? The following information is an introduction to the diagnostic value of laboratory assays and the predictive value theory, which involve concepts or procedures that are used to answer these questions.

II. There are four classifications of test results relative to the presence or absence of a disease.
   A. TP (true positive): a positive result that correctly identified a patient as having a specified disease
   B. TN (true negative): a negative result that correctly identified a patient as not having a specified disease
   C. FP (false positive): a positive result that incorrectly identified a patient as having a specified disease
   D. FN (false negative): a negative 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 criterion is used to separate a positive from a negative result? Is it a positive result when the result is above the appropriate reference interval? Or is it a positive result when the value exceeds a certain decision limit that could be within or outside of the reference interval?
   B. What determines if the animal does or does not have the disease of interest? Or 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 "pure gold standard" and thus the diagnostic value data must be considered to be relative to an imperfect standard.

IV. After test results are appropriately classified as TP, TN, FP, or FN, then several calculations are made to attempt to characterize the diagnostic properties or predictive value of the assay.

$$\text{Diagnostic sensitivity (as \%)} = \frac{\text{number of true positive}}{\text{number with specified disease}} = \frac{\text{TP \#}}{\text{TP \#} + \text{FN \#}} \times 100 \quad \textbf{(1.2.a.)}$$

$$\text{Diagnostic specificity (as \%)} = \frac{\text{number of true negative}}{\text{number without specified disease}} = \frac{\text{TN \#}}{\text{TN \#} + \text{FP \#}} \times 100 \quad \textbf{(1.2.b.)}$$

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$$\text{Diagnostic accuracy (as \%)} = \frac{\text{number correctly classified}}{\text{number of animals in study}} = \frac{\text{TP \#+TN \#}}{\text{TP \#+FP \#+TN \#+FN \#}} \times 100 \quad \textbf{(1.2.c.)}$$

$$\text{Predictive value of positive test (as \%)} = \frac{\text{number of true positive}}{\text{all positive results}} = \frac{\text{TP \#}}{\text{TP \#+FP \#}} \times 100 \quad \textbf{(1.2.d.)}$$

$$\text{Predictive value of negative test (as \%)} = \frac{\text{number of true negative}}{\text{all negative results}} = \frac{\text{TN \#}}{\text{TN \#+FN \#}} \times 100 \quad \textbf{(1.2.e.)}$$

A. Diagnostic sensitivity
   1. Definition: frequency with which a test is positive in patients that have the disease of interest (Eq. 1.2.a)
   2. A test that has high diagnostic sensitivity is a good test for screening for the presence of a disease because it has very few FN results. If the animal has the disease, there is a high probability that the test will be positive.

B. Diagnostic specificity
   1. Definition: frequency with which a test is negative in patients that do not have the disease of interest (Eq. 1.2.b)
   2. A test that has high diagnostic specificity can be a good test for confirming that an animal has a disease because it has very few FP results. If the result is positive, there is a high probability that the animal will have the disease.

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

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

E. PV(-), the predictive value of a negative test
   1. Definition: probability that a negative test result indicates that the animal does not have the disease (Eq. 1.2.e)
   2. A test that has a high PV(-) 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 is the prevalence of the disease in the studied population? The effects of prevalence will be illustrated in an example below. Basically, when the disease prevalence is very low, it is more likely that there will be FP results. Conversely, when the disease prevalence is very high, it is more likely that there will be FN results.

B. What method is used as the gold standard method for establishing the presence or absence of disease? Having an excellent gold standard is sometimes very difficult for spontaneous diseases and thus comparison against a poor gold standard may lead to questionable results. Also, consider this question: What would be the results of a comparison study if the new assay is actually better than the existing gold standard?

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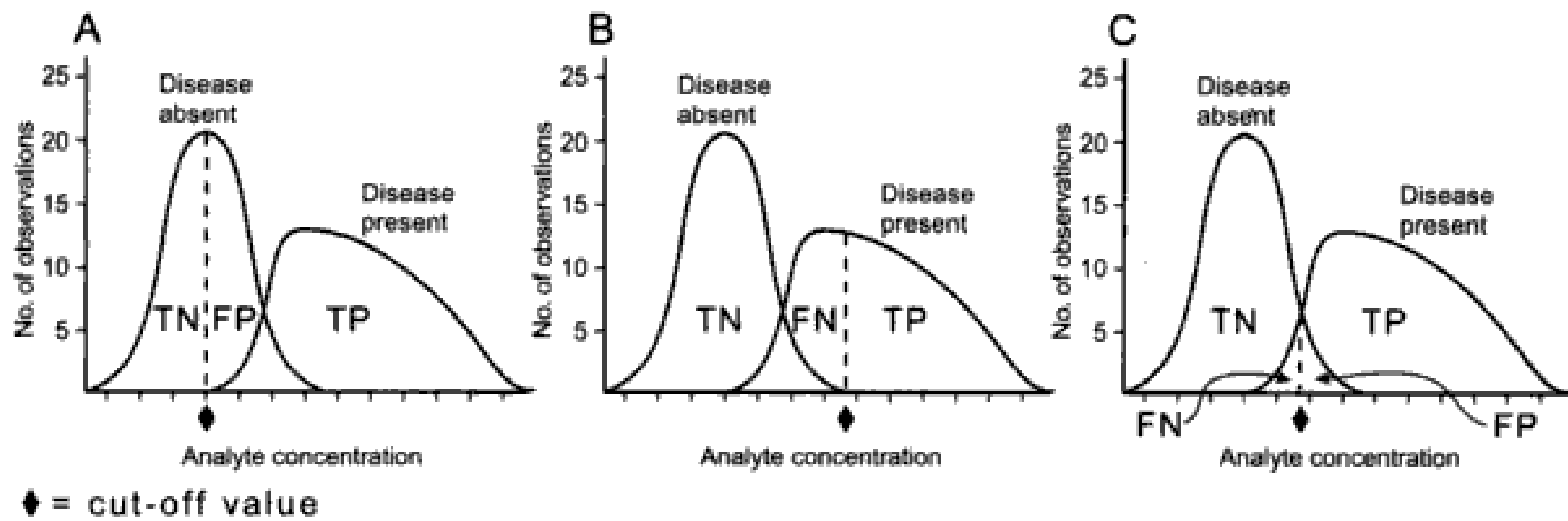

Fig. 1.4. Effects of different cutoff values on classifying assay results.

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 exist in real studies. The distribution data in the diseased group are not Gaussian; typically, such data are not Gaussian but may not be skewed as shown in this example.

Graph A: The cutoff value is near the mean analyte concentration found in the animals without the disease. With such a cutoff value,

- The diagnostic sensitivity would be 100% because there are no FN results.
- The diagnostic specificity would be 50% since there are equal numbers of TN and FP.
- The diagnostic accuracy would be poor because of the many FP results.
- The PV(+) would be poor because of the many FP results.
- The PV(-) would be 100% since there are no FN results.

Graph B: The cutoff value is at the highest value found in the animals without the disease.

- The diagnostic sensitivity would be poor (about 60%) since there are relatively many FN results.
- The diagnostic specificity would be 100% since there are no FP results.
- The diagnostic accuracy would be poor because of the many FN results.
- The PV(+) would be 100% since there are no FP results.
- The PV(-) would be poor because there are many FN results.

Graph C: The cutoff value is at a concentration where the least overlap between the groups occurs.

- This cutoff value 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 PV(+) and PV(-) would be high but not 100%.

C. What is going to be the cutoff value that separates a positive result from a negative result? Extensive evaluation of an assay is sometimes needed to find the best cutoff value. The effects of changing the cutoff value are illustrated in Fig. 1.4.

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 Fig. 1.5, assume the diagnostic sensitivity of a test is 90% and its diagnostic specificity is 80%.

1. In Example A of Fig. 1.5, with a disease prevalence of 30%
   a. the PV(+) is 66%; for all positive test results, 66% will be TP results.
   b. the PV(-) is 95%; for all negative test results, 95% will be TN results.
2. In Example B of Fig. 1.5, with a disease prevalence of 1%
   a. the PV(+) is 4.3%; for all positive test results, 4.3% will be TP results.
   b. the PV(-) is 99.9%; for all negative test results, 99.9% will be TN results.

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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

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

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

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

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

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

Fig. 1.5. Examples of diagnostic properties of assays.
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 assay's PV(+)? What is the assay's PV(-)?

- *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. Since the diagnostic sensitivity is 90%, then 90% of the 300 diseased dogs (270) will have a positive test result (TP) and 30 will have a negative test result (FN). Because the diagnostic specificity is 80%, then 80% of 700 dogs (560) will have negative results (TN) and 140 will have a positive result (FP).
- *Step 2:* Add the values for number of positive and negative results.
- *Step 3:* Calculate the PV(+) and PV(-) using Eq. 1.1 formulas.

In *Example B*, we discovered (via a gold standard) that 1% of 1000 dogs have the disease. Based on the prevalence, what is the assay's PV(+)? What is the assay's PV(-)?

- *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. Since the diagnostic sensitivity is 90%, then 90% of the 10 diseased dogs (9) will have a positive test result (TP) and 10% (1) will have a negative test result (FN). Because the diagnostic specificity is 80%, then 80% of 990 dogs (792) will have negative results (TN) and 198 will have a positive result (FP).
- *Step 2:* Add the values for number of positive and negative results.
- *Step 3:* Calculate the PV(+) and PV(-) using Eq. 1.1 formulas.

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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 PV(+) decreased from 66% to 4.3%. Thus, the PV(+) is less when the prevalence of the disease is lower.
   b. As the prevalence of the disease dropped from 30% to 1%, the PV(-) increased from 95% to 99.9%. Thus, the PV(-) 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.[10] 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]$_{ed}$.
   1. Gold standard for this study
      a. Cats were classified as having hyperthyroidism 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 T_3-suppression or TRH-stimulation test
      b. Cats were classified as not having hyperthyroidism if they did not meet hyperthyroidism criteria. The cats had clinical signs suggestive of hyperthyroidism (weight loss, vomiting, diarrhea, polyuria), but none had a palpable thyroid mass, none had increased [tT_4], and all had a diagnosis other than hyperthyroidism.
   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. Cutoff values represented the URL 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 cats, 837 had an increased [tT_4] and 903 had increased [fT_4]$_{ed}$.
      b. 221 cats did not have hyperthyroidism. Of these cats, none had increased [tT_4] and 14 had increased [fT_4]$_{ed}$.
   4. From the provided data, 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 (Fig. 1.6).
   5. Based on the evaluation of reported data and application of the above gold standard and cutoff values, these conclusions can be drawn.
      a. Serum [fT_4]$_{ed}$ had better diagnostic sensitivity (98%) than 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 will have increased [fT_4]$_{ed}$ than increased [tT_4].
      b. Serum [tT_4] had better diagnostic specificity (100%) than serum [fT_4]$_{ed}$ (94%). Thus, an increased serum [tT_4] is more indicative of feline hyperthyroidism than an increased serum [fT_4]$_{ed}$; i.e., [tT_4] had fewer FP results.
      c. Serum [fT4]$_{ed}$ had better diagnostic accuracy (98%) than serum [tT_4] (93%). Thus, if only [tT_4] or [fT_4]$_{ed}$ can be determined, serum [fT_4]$_{ed}$ has a better chance

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[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

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

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

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

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

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

[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

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

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

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

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

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

Fig. 1.6. Analysis of the diagnostic properties of serum [tT$_4$] and [fT$_4$]$_{ed}$.

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 PV(+) for increased [tT$_4$] was 100% and it was 98% for [fT$_4$]$_{ed}$.
e. The PV(-) 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.

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f. Note that the results of such studies may vary with different assays, different populations, different gold standards, and different cutoff values.
   (1) [tT_4] was used to determine the presence or absence of hyperthyroidism. The gold standard used (or method of establishing 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 if 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 PV(+) of diagnostic tests and lowers the PV(-). Accordingly, the predictive values of [tT_4] and [fT_4]$_{ed}$ would probably be different in a nonreferral veterinary practice.

VII. Application of the above methods to evaluate and compare diagnostic methods requires careful planning, appropriate choices of the animal populations (diseased versus nondiseased), and availability of an excellent gold standard. A major 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. Such a weighting process frequently is not appropriate in the clinical decision process.

RECEIVER OPERATING CHARACTERISTIC (ROC) CURVES[11,12]

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 true-positive rate and false-positive rate.
   A. True-positive rate is equal to diagnostic sensitivity expressed as a decimal (e.g., when the diagnostic sensitivity is 90%, the true-positive rate is 0.9; results are positive in 9 of 10 diseased animals).
   B. False-positive rate is equal to 1 minus the diagnostic specificity expressed as a decimal (e.g., when the diagnostic specificity is 70%, the false-positive rate is $1 - 0.7 = 0.3$, and 3 of 10 nondiseased animals would have a FP result. When the diagnostic specificity is 100%, there would be no FP results and thus the false-positive rate would be 0.0.)
   C. Fig. 1.7 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 is dependent 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 disorders is evaluated. Conversely, comparison of a sick group versus a healthy group is probably not appropriate or needed (animal was defined as healthy without laboratory tests).
   C. The accuracy of the comparison is very dependent on the accuracy of the gold standard procedure to differentiate disease-present from disease-absent groups. As there are very few "pure gold" procedures, results of the comparison need to be interpreted accordingly.

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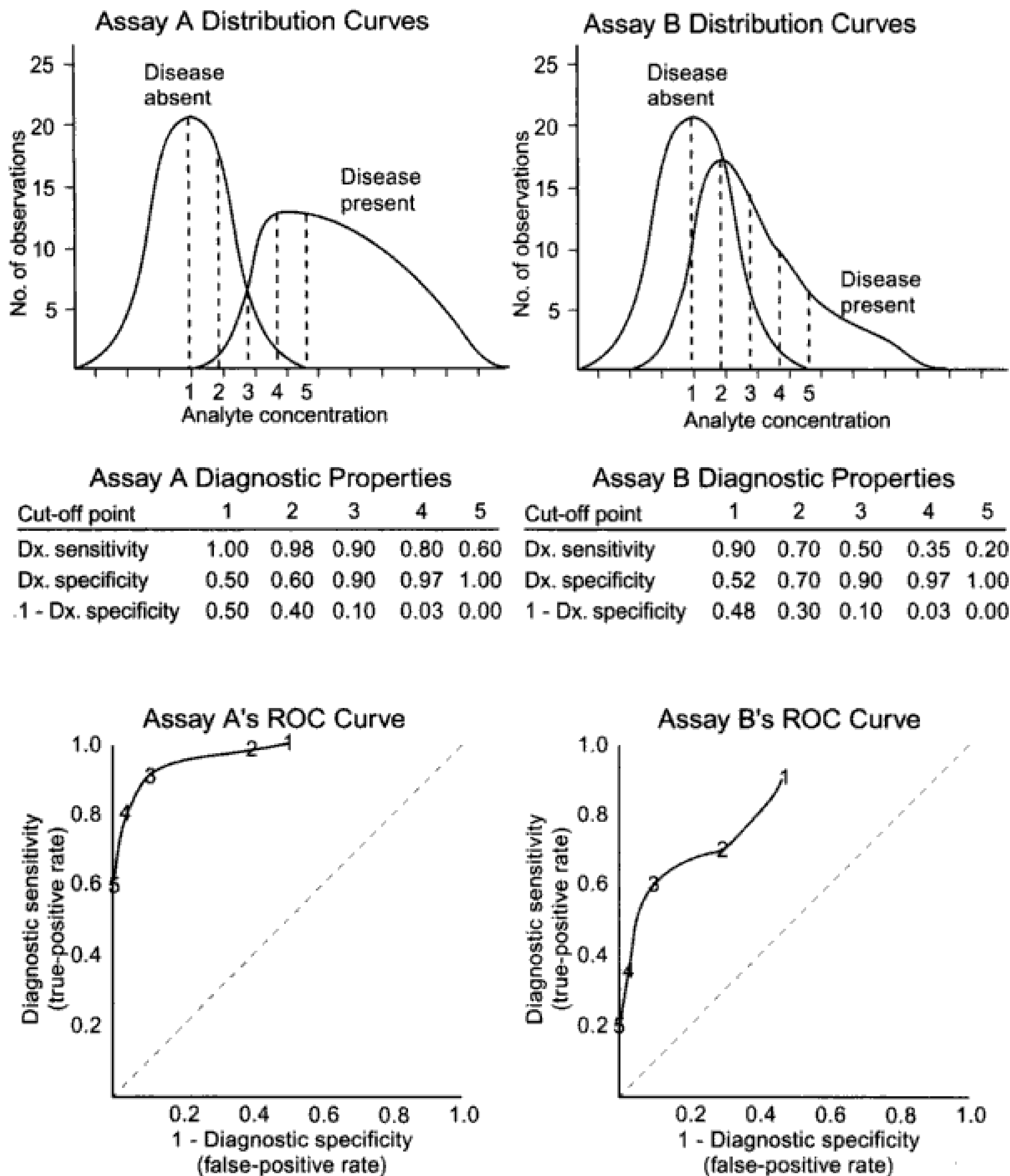

Assay A Diagnostic Properties

Cut-off point	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

Cut-off point	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

Fig. 1.7. 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). To gather data for a ROC curve, multiple cutoff points are selected that will provide different diagnostic sensitivity and specificity values. The cutoff points are then used to classify actual measured concentrations as being TP, FP, TN, or FN results. For the illustration in the top graphs, five cutoff points were selected and the dashed lines represent the separation of positive and negative results at each cutoff point.
- From the classified data, the diagnostic sensitivity (true-positive rate) and specificity values are calculated for each cutoff point (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 the same). The false-positive rate is calculated by subtracting diagnostic specificity from 1.
- The decimal fractions for true-positive rate and false-positive rate are plotted (lower graphs). 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 upper left corner of the graph (i.e., where nearly all positive results are TP results). In this comparison, Assay A is a better diagnostic procedure than Assay B for detecting a certain disease.

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Chapter 2

BASIC HEMATOLOGIC ASSAYS

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Table 2.1. Abbreviations and symbols in Chapter 2

[x]	concentration of x; x = analyte
× g	times standard gravity (9.80665 m/s^2)
C3	complement 3rd component
Ca^{2+}	calcium
CBC	complete blood count
CRP	corrected reticulocyte percentage
DNA	deoxyribonucleic acid
ECF	extracellular fluid
EDTA	ethylenediaminetetraacetic acid
ESAIg	erythrocyte surface-associated immunoglobulin
Hct	hematocrit
Hgb	hemoglobin
K_2EDTA	dipotassium ethylenediaminetetraacetic acid
K_3EDTA	tripotassium ethylenediaminetetraacetic acid
MCH	mean cell hemoglobin; mean corpuscular hemoglobin
MCHC	mean cell hemoglobin concentration; mean corpuscular hemoglobin concentration
MCV	mean cell volume; mean corpuscular volume
MPV	mean platelet volume
Na_2EDTA	disodium ethylenediaminetetraacetic acid
NMB	new methylene blue
nRBC	nucleated erythrocyte
PCV	packed cell volume
RBC	red blood cell; erythrocyte
RC	reticulocyte concentration
RDW	red blood cell distribution width
RMT	reticulocyte maturation time
RNA	ribonucleic acid
RP	reticulocyte percentage
RPI	reticulocyte production index
SI	Système International d'Unités
WBC	white blood cell; leukocyte
WRI	within reference interval

BLOOD SAMPLES AND SPECIMENS

I. Blood

A. Blood is composed of blood cells (erythrocytes, platelets, and five major leukocyte types) and plasma. Blood withdrawn from a blood vessel must immediately be mixed with an anticoagulant to prevent initiation of clot formation and to keep cells and other components in suspension.

B. Analysis or processing of whole blood must be relatively rapid because the cells die within a few hours and thus a sample will become 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.

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II. Plasma

A. Plasma is the fluid component of blood that is harvested after centrifugation of an anticoagulated blood sample. Plasma will contain the anticoagulant that can interfere with some assays.

B. Plasma has two major components.

1. Water: about 92%–95% of plasma volume. 100 mL of plasma contains 92–95 mL of H_2O.
2. 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.

III. Serum

A. Serum is the fluid component of blood that is harvested after centrifugation of a coagulated (clotted) blood sample.

B. 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.

C. During the clotting process, substances are released from cells that alter the analyte concentrations in serum. For example, platelets release K^+ and thus serum $[K^+]$ is higher than plasma $[K^+]$ (see Chap. 9).

D. Generally, the composition of serum or plasma is very similar to the composition of ECF.

IV. 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 Na_2EDTA, K_2EDTA or K_3EDTA)
   a. Preferred anticoagulant for almost all routine hematologic tests, including the CBC assays
   b. Binds Ca^{2+} and other divalent cations (Mg^{2+}, Cu^{2+}, Pb^{2+})
2. Citrate (as sodium citrate)
   a. Preferred anticoagulant for most tests of the coagulation system; Ca^{2+} is added to the citrated plasma to override the effects of citrate and allow coagulation enzymes to function.
   b. Because it has low toxicity, citrate is also preferred for collection of whole blood to be used for transfusions.
3. Oxalates (as lithium, ammonium, and potassium salts)
   a. Used for a few specialized laboratory tests
   b. Generally distort morphologic features of leukocytes and erythrocytes

B. Heparin (as lithium, ammonium, potassium, or sodium salts) activates antithrombin III, which then inhibits the activity of several coagulation factors (including thrombin). It also binds Ca^{2+}, but major action is through antithrombin III.

1. Used for several special laboratory assays (such as blood gas analysis) and can be used for many clinical chemistry assays
2. Major disadvantages
   a. Alters morphologic features and staining of leukocytes
   b. Allows clotting as effects are slowly overridden by coagulation system
   c. Allows platelet clumps to form

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COMPLETE BLOOD COUNT (CBC)

I. Purposes of the CBC
   A. To screen the hemic system for abnormalities or its response to a disease
   B. To confirm or define the presence of a hematologic disorder

II. Basic information obtained from results of a CBC
   A. If CBC results are WRI, the net effect of a disease on the hematopoietic system has been minimal.
   B. If a cell concentration is increased, the disease is causing at least one of the following:
      1. Increased production of that cell type
      2. A shift of that cell type from a storage or noncirculating pool to circulating blood
      3. An increased circulating life span of the cell type. The rate of cell loss to tissues or rate of cell death is decreased.
   C. If a cell concentration is decreased, the disease is causing at least one of the following:
      1. Decreased production of that cell type
      2. A shift of that cell type from the circulation to a noncirculating pool
      3. Decreased circulating life span of the cell type
   D. If morphologic features of a given cell type are abnormal, either (1) there is a defect in hematopoiesis that causes the production of abnormal cells, or (2) morphological abnormalities are acquired as the cell circulates in the body.

III. Major components of the CBC
   A. Erythrogram ("erythrocyte picture")
      1. Erythrocyte structure as seen on a stained blood film (see Chap. 4)
      2. Hct (synonym: PCV)
         a. If there are 100 mL of blood with a Hct of 45%, then erythrocytes occupy 45 mL.
         b. A Hct will accurately reflect the [RBC] in a blood sample if the MCV is WRI.
         c. Unit: vol % (commonly just %); 40 vol % = 0.40 (SI expression is a unitless decimal fraction)
      3. [Hgb]
         a. [Hgb] in blood is the grams of Hgb per 100 mL of blood. Essentially all Hgb in blood is in erythrocytes except in a few pathologic states (such as intravascular hemolysis causing hemoglobinemia).
         b. [Hgb] will accurately reflect [RBC] if the MCHC is WRI and hemoglobinemia is not present.
         c. Unit conversion: g/dL × 10 dL/L = g/L (SI unit, nearest 1 g/L)[1]
      4. [RBC]
         a. [RBC] is the number of erythrocytes per unit volume of blood (in clinical jargon, commonly referred to as RBC count).
         b. Unit conversion: $(\# \times 10^6/\mu L) \times 10^6\ \mu L/L = \# \times 10^{12}/L$ (SI unit)
      5. Wintrobe's erythrocyte indices: MCV, MCHC, and MCH
         a. Wintrobe's erythrocyte indices are three values that are used to characterize erythrocytes in peripheral blood. The erythrocyte indices form the basis of the morphologic classification of anemias (see Chap. 4).
            (1) MCV: volume per average erythrocyte expressed in femtoliters (fL) or cubic micrometers (μm^3)

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(2) MCHC: Hgb concentration per average erythrocyte expressed as grams of Hgb per 100 mL of erythrocytes (g/dL); g/dL × 10 dL/L = g/L (SI unit)
(3) MCH: quantity of Hgb per average erythrocyte expressed in picograms (pg)

b. In most blood specimens, not all erythrocytes are the same (i.e., the erythrocytes have different volumes, Hgb concentrations, and Hgb contents). It is important to remember that the MCV, MCHC, and MCH values represent the average values for all erythrocytes in the sample.

c. Relationship of indices
(1) Because MCH represents how much Hgb is in an average erythrocyte and MCV represents the volume of an average erythrocyte, the MCHC of an average erythrocyte can be calculated by dividing the MCH by the MCV (Eq. 2.1.a).

$$MCHC = \frac{MCH}{MCV} \quad \textbf{(2.1.a.)}$$

Example: MCH = 20 pg, MCV = 60 fL

$$MCHC = \frac{20\ pg}{60\ fL} = \frac{20\times10^{-12}\ g}{60\times10^{-15}\ L} = \frac{20,000\times10^{-15}\ g}{60\times10^{-15}\ L} = 333\ g/L = 33.3\ g/dL$$

$$MCV = \frac{Hct\times10}{[RBC]} \quad MCHC = \frac{[Hgb]\times100}{Hct} \quad MCH = \frac{[Hgb]\times10}{[RBC]} \quad \textbf{(2.1.b.)}$$

$$Hct = \frac{MCV\times[RBC]}{10} \quad \textbf{(2.1.c.)}$$

(2) When originally described by Wintrobe, the indices represented the calculated interrelationships of three measured values: Hct (reported as a %), [Hgb] (reported as g/dL), and [RBC] (reported as $10^6/\mu L$) (Eq. 2.1.b).

d. The Wintrobe formulas are used to calculate some of the results generated by impedance cell counters.
(1) Hct is calculated from the measured MCV and [RBC] (Eq. 2.1.c).
(2) MCHC is calculated from the measured [Hgb] and calculated Hct (Eq. 2.1.b).
(3) MCH is calculated from the measured [Hgb] and measured [RBC] (Eq. 2.1.b).

e. The Technicon (ADVIA®) analyzers calculate the MCHC as above but also measure MCHC by analysis of light scatter caused by Hgb in erythrocytes.

f. Relationship of blood [Hgb] and MCHC
(1) Sample 1: Hct = 50%, [Hgb] = 15 g/dL
(a) If you have 100 mL of blood with a Hct of 50% and a [Hgb] of 15 g/dL (grams of Hgb per 100 mL of blood), then you have 50 mL of erythrocytes, 50 mL of plasma, and 15 grams of Hgb in the erythrocytes.
(b) Therefore, you have 15 grams of Hgb per 50 mL of erythrocytes or 30 grams of Hgb per 100 mL of erythrocytes, and thus the MCHC = 30 g/dL.
(2) Sample 2: Hct = 33%, [Hgb] = 10 g/dL
(a) If you have 100 mL of blood with a Hct of 33% and a [Hgb] of 10 g/dL (grams of Hgb per 100 mL of blood), then you have 33 mL of erythrocytes, 67 mL of plasma, and 10 grams of Hgb in the erythrocytes.
(b) Therefore, you have 10 grams of Hgb per 33 mL of erythrocytes or 30 grams per 100 mL of erythrocytes, and thus the MCHC = 30 g/dL.

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(3) Relationship of Hgb and Hct (with conventional units)
  (a) When the MCHC is 33.3 g/dL, the Hgb values will be one-third of the Hct value (e.g., Hgb = 15 g/dL and Hct = 45%; or Hgb = 8 g/dL and Hct = 24%).
  (b) Because the MCHC values in most blood samples are about 32–36 g/dL, the Hgb value typically will be about one-third of the Hct value.

B. Leukogram ("leukocyte picture")
1. Leukocyte structure as seen on a stained blood film (see Chap. 3)
2. [WBC]
   a. [WBC] is the number of leukocytes per unit volume of blood (in clinical jargon, commonly referred to as WBC count).
   b. By some methods, the [WBC] actually is a total nucleated cell concentration. If it is, the [WBC] must be corrected when nRBCs are present (see Calculations Involving Nucleated Erythrocytes, p. 45]
   c. The [WBC] by itself is of limited value without assessing the concentrations of each type of leukocyte.
   d. Unit conversion: $\#/\mu L \times 10^6\ \mu L/L = \# \times 10^6/L$ (SI unit)
3. WBC differential count
   a. A WBC differential count is done by differentiating 100 or more consecutive leukocytes on a stained blood film. Results of the differential count are percentages. A WBC differential count should be done in a blood film's "counting window" (i.e., that part of a blood film where there is a monolayer of erythrocytes that occasionally touch each other and where the leukocytes lie flat enough for their nuclear and cytoplasmic features to be distinct).
   b. A WBC differential count is only an estimate of the leukocyte percentages in a blood sample because only a small portion of the leukocytes in the blood sample is differentiated. A sample of 100 cells will not be consistently representative of the whole leukocyte population.
   c. Rule of thumb: Differentiate 100 leukocytes for every 10,000 leukocytes/µL to obtain representative percentages.
4. Concentrations of each type of leukocyte
   a. With traditional methods, individual leukocyte concentrations are determined by multiplying the [WBC] by the leukocyte percentages obtained from the WBC differential count.
   b. Because of the relatively poor reproducibility of the WBC differential count, the calculated values should be considered approximate concentrations.

C. Assessment of platelets
1. Structure of platelets as seen on a stained blood film (see Chap. 5)
2. Platelet concentration
   a. Platelet concentration is the number of platelets per unit volume of blood (in clinical jargon, commonly referred to as platelet count).
   b. Unit conversion: $(\# \times 10^3)/\mu L \times 10^6\ \mu L/L = \# \times 10^9/L$ (SI unit)
3. MPV: volume per average platelet expressed in femtoliters (fL) or cubic micrometers (μm^3)

IV. Blood sample for CBC
A. Blood is collected into a tube containing either Na_2EDTA or K_3EDTA and immediately mixed by slowly inverting the tube at least 10 times. To obtain accurate results, the sample must be free of clots and platelet clumps.

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B. Platelets can be easily activated during blood collection; activation results in clumping. Platelet clumping can occur because of hyperactive platelets, slow or poor venipuncture technique, and delayed or inadequate mixing of blood and EDTA. Platelets of cats and cattle are very prone to clumping.

C. Stability of cells
   1. [RBC] and [WBC] are usually considered to be stable for several hours at 25°C and for up to 24 hr at 4°C.
   2. Platelet concentrations are reportedly stable for 5 hr at room temperature and 24 hr when samples are refrigerated (4°C).[2] On average, platelet concentrations remained relatively stable in EDTA-anticoagulated canine blood for 48 hr (kept at room temperature for the first 8 hr and refrigerated for the following 40 hr), but there were considerable increases and decreases in platelet concentrations of individual samples.[3]
   3. Morphologic features of erythrocytes and platelets are usually stable for at least as long as their concentrations are stable. Morphologic changes can be seen in leukocytes within an hour of sample collection. These time-related changes include cytoplasmic vacuolation, swollen or hyalinized nuclei, cell lysis, pyknosis, and karyorrhexis.

HEMATOLOGIC INSTRUMENTS AND METHODS

I. Impedance cell counters (e.g., Coulter counters, Baker System, Cobas Minos ST-Vet, Mascot Multispecies Hematology System, CELL-DYN®, Heska™ Vet ABC–Diff Analyzer)

A. Basic principles
   1. When a nonconductive particle (e.g., cell) passes through an aperture, it creates an electrical interference in a current that is flowing through a conductive liquid.
   2. The number of particles detected within a defined volume of diluted blood represents a cell (particle) concentration. The degree of interference is dependent on the apparent volume of the particle (shape may influence), and apparent volumes are used to differentiate particles. The volume of each particle is recorded so that an average volume can be calculated.
   3. Most impedance cell counters were designed to evaluate human blood cells and need to be modified to evaluate domestic mammal blood. Small erythrocytes of some species (e.g., goats, sheep, and some horses) are too small to be reliably detected. Also, large platelets (as commonly seen in cats) may be counted as erythrocytes and not platelets. Lysing solutions and procedures may also need modification because of species differences in susceptibility to cell lysis.

B. Typically, blood is processed along two paths.
   1. Erythrocyte path: MCV, [RBC], and platelet concentrations are determined in diluted blood.
   2. Leukocyte path: [WBC] and [Hgb] are determined in diluted blood after cells (leukocytes and erythrocytes) are lysed but nuclei remain as particles.

C. Measured values
   1. MCV: average volume of particles (mostly erythrocytes) that are larger than a minimal threshold setting
   2. [Hgb]: spectrophotometric assay for Hgb after erythrocyte lysis
   3. [RBC]: concentration of particles (mostly erythrocytes) that are larger than a minimum threshold setting (e.g., > 30 fL) and smaller than a maximum threshold setting. Some instruments correct for the presence of leukocytes.
   4. [WBC]: concentration of particles (nuclei) present after blood cells are lysed

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5. Platelet concentration: concentration of particles that are within a defined volume interval (e.g., 2–30 fL)
6. MPV: average volume of particles (mostly platelets) that are within a defined volume interval

D. Calculated values (formulas appropriate for traditional units)
1. Hct is calculated from the rearrangement of the Wintrobe MCV formula (Eq. 2.1.c).
2. MCHC and MCH values are calculated with the traditional MCHC and MCH formulas (Eq. 2.1.b).
3. RDW
   a. RDW is an estimate of the degree of anisocytosis in a blood sample. The greater the variability in erythrocyte volumes, the greater the RDW, and thus the greater the degree of anisocytosis. A high RDW could occur due to the presence of more macrocytes, more microcytes, or more of both.
   b. Instruments may use different RDW formulas and the fraction of erythrocytes included in the calculation also varies. The CELL-DYN® evaluates the frequency distribution plot of erythrocyte volumes and calculates the coefficient of variation (CV), which is reported as a percentage.

II. Optical or laser flow cell cytometers (e.g., CELL-DYN® instruments, Technicon H1 [ADVIA®])

A. Basic principles
1. Laser light is scattered when it hits a cell. The type of scatter is dependent on cell size, internal structure, granularity, and surface structure. The type of light scatter is used to differentiate cells.
2. Instruments may be able to differentiate the major leukocytes, but computer programs must be specific for each species and abnormal leukocytes may not be correctly classified.

B. Optical counters also have the components of impedance counters (see I in this section).

III. Conductivity methods (i-STAT®, Stat Profile® M Analyzer)

A. Sensors in silicon chips detect changes using potentiometric, amperometric, or conductometric principles.

B. The amount that erythrocytes reduce the conductivity of a fluid is dependent on the percentage of blood volume occupied by erythrocytes. Thus, reduction in conductivity is related to Hct. Markedly increased blood concentrations of proteins, leukocytes, or lipids will also reduce the conductivity and thus give falsely increased Hct values.

C. [Hgb] is calculated by multiplying the Hct decimal fraction (e.g., 0.40) by an assumed MCHC value (e.g., 34 g/dL).

IV. Centrifugation analysis

A. Microhematocrit tube method
1. Hematocrit: *hemato-* (blood) *-crit* (denoting separation)
2. When first described, the term *hematocrit* was the name of the procedure used to separate blood into its major components: packed erythrocytes, buffy coat layer, and plasma. With common use of the procedure, hematocrit started to indicate the major result of the procedure (i.e., Hct or PCV). Today, the terms *hematocrit* and *packed cell volume* are frequently used as synonyms. When a small capillary tube was intro-

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duced for the procedure, it became the *microhematocrit* method to differentiate it from the larger Wintrobe hematocrit tubes.

3. High-speed centrifugation (about 13,000 × g) 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 nucleated erythrocytes (listed from least to most dense). The buffy coat cells typically occupy about 0.5 to 1% of the blood volume.
4. Because of potential inaccuracies of measuring [RBC] and MCV in domestic mammals, the microhematocrit tube method is generally considered the "gold standard" method of determining blood Hct.

B. Quantitative buffy coat (QBC) analysis,[4,5] QBC® VetAutoread™

1. A special capillary tube that contains a plastic cylinder or float is used. Because of the cylinder's density, it will float in the region of the buffy coat cells with centrifugation. Because of the cylinder's diameter, the length of the buffy coat is expanded around the float (Fig. 2.1). Fluorescence is used to differentiate cell layers on the basis of lipoprotein, RNA, and DNA contents.
2. 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 will correlate 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.
3. Results generated by the IDEXX QBC® VetAutoread™ hematology system and the basic principles of each evaluation
   a. Hct is determined by the volume of blood occupied by erythrocytes after centrifugation (analogous to microhematocrit tube method).

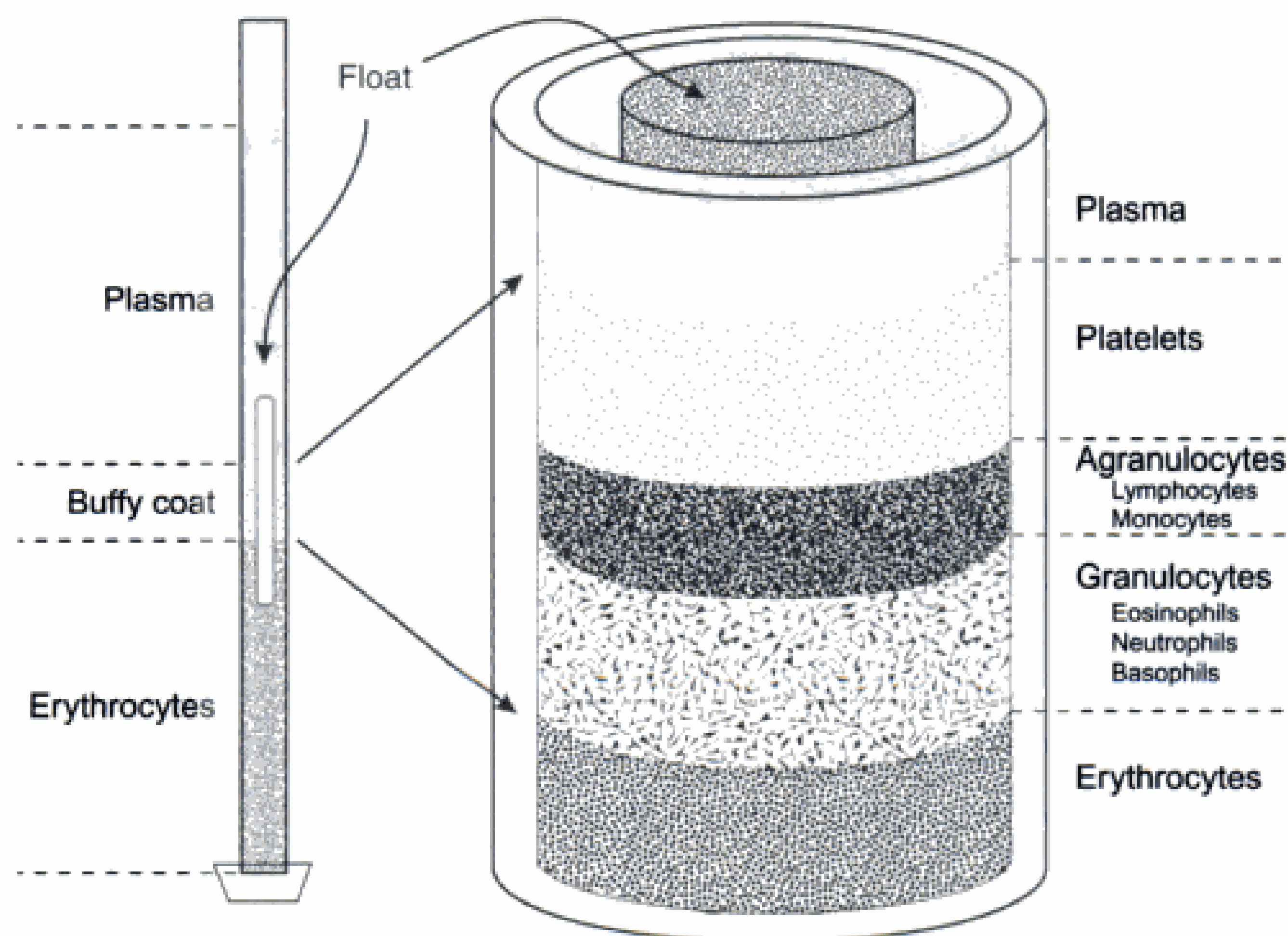

Fig. 2.1. Schematic representation of IDEXX QBC® VetTube after centrifugation of whole blood. Centrifugal forces separate the components of blood into five layers (plasma, platelets, agranulocytes, granulocytes, and erythrocytes) based on their relative densities.

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b. MCHC is determined by a relationship between density of erythrocytes and the density of the plastic float. The lower the MCHC, the deeper the plastic float will sink into the packed erythrocytes.
c. [Hgb] is calculated from the Hct and MCHC values.
d. Reticulocyte percentage is determined by the thickness of an RNA fluorescent layer near the top of the erythrocyte layer (bottom of buffy coat).
e. nRBC are detected by the presence of DNA fluorescence near the top of the erythrocyte layer.
f. Total [WBC] is calculated by adding the granulocyte and combined lymphocyte/monocyte concentrations.
   (1) Granulocyte concentration is determined by the thickness of the cell layer with characteristic DNA and RNA/lipoprotein fluorescence. 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.
   (2) 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.
g. Platelet concentration is determined by the thickness of the cell layer above the lymphocyte/monocyte layer that has characteristic RNA/lipoprotein fluorescence.
h. Fibrinogen concentration is estimated by application of the heat precipitation principle (see Chap. 7) with the thickness of the precipitated fibrinogen band determining the fibrinogen concentration.

MICROSCOPIC EXAMINATION OF STAINED BLOOD FILMS

I. Microscopic examination of a stained blood film should always be a part of a CBC, even if an instrument provides an automated leukocyte differential count. A detailed description of the preparation, staining, and examination of the blood film is beyond the scope of this chapter. The basic components of the processes are as follows:
   A. A blood film is made to obtain an even distribution of cells and an adequate "counting window" (i.e., that part of the smear where there is a monolayer of erythrocytes that occasionally touch each other and where nuclear and cytoplasmic features of leukocytes are distinct).
   B. The air-dried blood film is stained with a Romanowsky-type stain (e.g., Wright, Wright-Giemsa, Diff-Quik, Quik-Dip) that provides differential staining of cells.
   C. A blood film examination includes scanning with 4× or 10× objectives and more critical evaluation with high-dry (40×) or oil objectives (50× or 100×).
      1. Are the cells evenly distributed and properly stained?
      2. Are there abnormal large structures in the blood (frequently concentrated in the feathered edge) such as microfilaria, platelet clumps, macrophages, epithelial cells, endothelial cells, or megakaryocytes? If so, record their presence.
      3. Do the erythrocyte and leukocyte densities correspond with the known cell concentrations in the sample? If not, check the accuracy of the cell concentrations.
      4. Estimate platelet density in several 1000× oil fields and compare with expected values; record the presence of giant or shift platelets (platelets larger than erythrocytes in most species) or platelet inclusions.
      5. Evaluate erythrocytes for abnormal shapes, sizes, colors, or inclusions.

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6. Complete a leukocyte differential count (see below) and record. Evaluate observed leukocytes for morphologic abnormalities and record findings.

II. Microscopic examination of a stained blood film is especially important in animals with abnormal concentrations of leukocytes, erythrocytes, or platelets. Defects found in the blood cells are described in Chapters 3, 4, and 5.

III. Staining of blood cells
   A. Romanowsky stains are the best for staining blood cells. Romanowsky (1891) described a combination of eosin and methylene blue to produce a spectrum of colors from blue to reddish-orange depending on the pH of the cell's content.
      1. Wright stain: combination of eosin and oxidized methylene blue; the oxidized methylene blue stains are called Azure dyes.
      2. Other Romanowsky stains include Giemsa, Wright-Giemsa, and Wright-Leishman that are various combinations of Azure dyes and eosin.
      3. Generally when stated or written, "Wright" stain refers to a Romanowsky-type stain and not the original Wright stain.
   B. What are the contents of cells that are stained?
      1. Acidic structures (e.g., DNA and RNA) attract the basic Azure dyes, which stain structures various colors of purple to blue.
      2. Alkaline structures (e.g., Hgb, 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), *philic* (loving)
      2. *Eosinophilic:* loves eosin (acidic) dye; will have red to orange colors
      3. *Basophilic:* loves basic (alkaline, Azure) dyes; will be blue to purple colors
      4. *Azurophilic:* loves Azure dye; will have a blue to purple to reddish-purple to pink color depending on the substance's pH

IV. Estimate of platelet concentration on blood films
   A. Platelet estimation is a common routine method of platelet enumeration in blood. Platelet estimations are frequently done, when automated values are unreliable, so that a time-consuming hemocytometer platelet method does not have to be done. There are several methods of estimating platelet concentrations on Wright-stained blood films. Methods are valid only if platelet clumps are not present in the blood sample.
   B. Procedure
      1. Scan the feathered edge and smear body for platelet clumps. Find the appropriate "counting window." Using the 100× oil objective, again assess for platelet clumping. If platelets are clumped, report "clumped platelets."
      2. If platelets are well distributed, estimate the average number of platelets per 1000× field. Enough fields should be evaluated to generate a representative assessment; five independent fields may be used as a guideline, but more fields may be required with decreased concentrations. Results are probably most critical with moderate to marked thrombocytopenias. Use the following table of average platelet number per 1000× field to report estimated platelet concentrations (Table 2.2).
      3. The conversion factor (1 platelet/1000× field equals about 20,000 platelets/µL) was chosen because the data supporting it are published and it will result in fewer false suggestions of thrombocytopenia than would occur with the other commonly mentioned 1 to 15,000/µL conversion. If the 15,000/µL conversion is actually more

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Table 2.2. Estimation of platelet concentration on a blood film

Species	Markedly decreased	Moderately decreased	Mildly decreased	WRI	Possibly increased	Increased
Canine	≤ 3[a]	4–7	8–9	10–25	26–30	> 30
Feline	≤ 3	4–11	12–14	15–40	41–48	> 48
Equine	≤ 2	3	4	5–17	18–21	> 21
Bovine	≤ 2	3	4	5–40	41–48	> 48
Ovine	≤ 3	4–9	10–12	13–37	38–45	> 45
Caprine	≤ 3	4–11	12–14	15–30	31–36	> 36

[a] Average number of platelets found in 1000× microscopic field (100× objective times 10× ocular) in the "counting window."

accurate, mild thrombocytopenias will be missed, and thrombocytoses may be falsely suggested by the 1 to 20,000 method.

4. The area represented by a 1000× microscopic field varies with the optics of microscopes (e.g., the observed field in a wide-field ocular will be larger than a regular ocular). Therefore, the conversion factor can vary as much as 30% between microscopes. For example, the 1:20,000 method may be appropriate for regular oculars and the 1:15,000 method may be more appropriate for wide-field oculars.

RETICULOCYTES

I. Major concepts
   A. Reticulocytes are non-nucleated, immature erythrocytes with stainable cytoplasmic RNA.
      1. On an NMB-stained blood film, the RNA appears as punctate or reticulated basophilic structures.
      2. On a Wright-stained blood film, the RNA will give the polychromatophilic erythrocyte its blue or basophilic tinctorial properties.
   B. Reticulocytes of nonanemic dogs and people have life spans of 1–2 days. The life spans of cat reticulocytes are more variable (see II.B.2 in this section). In health, cattle and horses do not have circulating reticulocytes.
   C. Reticulocytosis: increased RC, CRP, or polychromasia
      1. In species other than horses, a reticulocytosis is the best semiquantitative evidence of increased erythropoiesis. Horses very, very rarely have circulating blood reticulocytes.
      2. In cattle after acute blood loss, a reticulocytosis is expected within 3–4 days and peak production is expected in 7–14 days. A few large or shift reticulocytes may be seen in the blood before a reticulocytosis is present.[6]
      3. The response in dogs 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 days.
      4. In cats after acute removal of erythrocytes by repeated phlebotomies to create a Hct value of 50% of baseline values:[7]
         a. An aggregate reticulocytosis occurred by day 2, peaked at day 4, and returned to baseline values by day 9 even though the cats were still anemic (Hct values in low 20s).
         b. A slight punctate reticulocytosis was present by day 1, a peak reticulocytosis occurred from 7 through 14 days, and punctate RP values did not return to baseline until after Hct returned to pre-phlebotomy values (after 3 weeks).

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5. The degree of increased polychromasia should correspond to the degree of aggregate reticulocytosis.

II. Types of reticulocytes
  A. In most species, any non-nucleated erythrocyte that contains RNA (stainable with NMB or other vital stains) is called a reticulocyte.
  B. In cats, reticulocytes stained by NMB may be classified by two systems. The punctate/aggregate system is the more commonly used.
    1. Punctate and aggregate reticulocytes[8]
      a. Punctate: cells with 2 to 6 small granules of reticulum
      b. Aggregate: cells with large aggregates of reticulum
      c. Degree of polychromasia seen on a Wright-stained smear tends to correlate with the aggregate RP.[7]
    2. Type I, II, and III reticulocytes[9,10]
      a. Type I: oldest form. Cells are uniform in size and stain light green with faint blue stippling. Circulating life span is 3 days; maximum concentration occurs about 10–12 days after onset of anemia.
      b. Type II. Cells vary somewhat in size and stain light green with large dark granules. Circulating life span is about 12 hr; maximal concentration occurs about 4 days after onset of anemia in an otherwise healthy cat.
      c. Type III: youngest form. Cells generally are larger than nonreticulated cells and have blue-green cytoplasm and a heavy dark blue granular network. Circulating life span is 12 hr; maximal concentration occurs about 4 days after onset of anemia.

III. Reticulocyte percentage (RP) (also called reticulocyte count)
  A. Definition: percentage of erythrocytes that are reticulocytes in a blood sample. For example, if there are 10 reticulocytes per 1000 erythrocytes, the RP is 1.0%.
  B. Basics of procedures
    1. Manual procedure
      a. 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). NMB penetrates an erythrocyte and stains (and precipitates) its cytoplasmic RNA. A smear of the blood-NMB mixture is made and air-dried for microscopic examination.
      b. The number of reticulocytes observed while counting 1000 non-nucleated erythrocytes is recorded. For cats, some laboratories only count the aggregate reticulocytes; other laboratories count aggregate and punctate reticulocytes and report both.
    2. Automated techniques
      a. QBC®-VetAutoread™: Canine RP is estimated by determining the thickness of RNA fluorescent cells that are at the top of the erythrocyte layer and on bottom of the granulocyte layer.
      b. Flow cytometer methods
        (1) In the CELL-DYN® and after staining with NMB and sphering in a special diluent, reticulocytes are enumerated by a unique light scatter created from precipitated ribosomal RNA. RP is determined by the percentage of erythrocytes with the light-scattering property.
        (2) In the flow cytometers and after staining reticulocytes with a fluorescent nucleic acid-binding dye (thiazole orange), erythrocytes pass through the instrument that identifies fluorescent and nonfluorescent cells. RP is determined by the percentage of erythrocytes that are fluorescent.

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C. An increased RP does not always indicate the presence of a reticulocytosis, especially in moderate to severe anemias (see Table 2.3). Conversely, a marrow may be responding to a mild anemia without generating an increased RP because the reticulocytes mature in the marrow before release and thus reticulocytosis is not seen but the hematocrit steadily climbs.

D. Microscopic determination of RP has relatively poor analytical precision; therefore, RP should be considered an estimate. Values that are calculated using the RP (i.e., RC, CRP, RPI) should also be considered estimates.

E. The degree of increased polychromasia should correspond to the increase in RP (except for punctate reticulocytes).

IV. Reticulocyte concentration (RC) (also called absolute reticulocyte count)

A. Definition: concentration of reticulocytes in blood expressed as number of reticulocytes per µL (or L) of blood.

B. RC equation (Eq. 2.2.a).

$$RC = RP \times [RBC] \quad \textbf{(2.2.a.)}$$

$$CRP = RP \times \frac{\text{patient's Hct}}{\text{average Hct for species}} \quad \textbf{(2.2.b.)}$$

$$RPI = \frac{CRP}{RMT} \quad \textbf{(2.2.c.)}$$

V. Corrected reticulocyte percentage (CRP) (also called absolute reticulocyte percent and absolute % reticulocyte count)

A. Calculation of CRP converts RP to a percentage that would estimate the RP if the animal were not anemic.

B. CRP equation (Eq. 2.2.b).

C. In theory, the reference interval for CRP should be the same as the RP reference interval. Therefore, if a CRP is greater than the reference interval for that for RP, there is a reticulocytosis.

D. Just as the RP must be corrected for the severity of the anemia, the degree of increased polychromasia needs to be interpreted with knowledge of the Hct.

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

A. During accelerated erythropoiesis, younger reticulocytes (called shift reticulocytes) may be released and they may have longer circulating life spans before becoming mature erythrocytes. Thus, the CRP may be increased due to increased life span of reticulocytes and not necessarily increased production. Some people recommend the CRP be adjusted for the prolonged life spans by calculating the RPI.

B. Equation for RPI (Eq. 2.2.c).

1. Application of the RPI requires that RMT values be known for each species in health and during accelerated erythropoiesis. RMT values frequently written for human reticulocytes are as follows: When Hct = 45%, RMT = 1.0 day; Hct = 35%, RMT = 1.5 days; Hct = 25%, RMT = 2.0 days; Hct = 15%, RMT = 2.5 days.

2. Very few RMT values for domestic animals are available.

a. In healthy dogs, reported values include a mean of 31 hr, range of 19–43 hr;[10] values for anemic dogs are not known but probably are longer.

b. In cats, reported values are 12 hr for aggregate reticulocytes and 72 hr for punctate reticulocytes.

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C. Until the validity and value of the RPI is established, the CRP or RC should be used to assess regenerative status. When interpreting the calculated values in an anemic animal, the possibility of longer RMT should be considered.

VII. Evaluation of reticulocyte values (see Table 2.3)

CALCULATIONS INVOLVING NUCLEATED ERYTHROCYTES (nRBC)

I. Correction of [WBC] for the presence of nucleated erythrocytes (nRBC) if the [WBC] is really a total nucleated cell concentration
   A. Manual and some electronic methods determine [WBC] by counting nucleated cells or nuclei (either of leukocytes or nRBCs). By these methods, a measured [WBC] represents the sum of nucleated cell concentrations (leukocytes plus nRBC).
   B. If nRBC are observed during the examination of the blood film, they are enumerated by counting the number of nRBC seen while 100 leukocytes are differentiated and counted. The conventional method of recording the number of nucleated erythrocytes is number of nRBC/100 WBC (e.g., 10 nRBC/100 WBC).
   C. Rule of thumb: The measured [WBC] is corrected for the presence of nRBC if there are 10 or more nRBC/100 WBC. The error in the measured [WBC] is usually clinically insignificant when there are less than 10 nRBC/100 WBC. However, the correction may be done with fewer than 10 nRBC/100 WBC.
   D. Corrected [WBC] equation (Eq. 2.3.a).

$$\text{corrected [WBC]} = \text{measured [WBC]} \times \frac{100}{100 + \#\,\text{nRBC}/100\text{WBC}} \qquad \textbf{(2.3.a.)}$$

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

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

$$\text{[nRBC]} = \text{measured [WBC]} - \text{corrected [WBC]} \qquad \textbf{(2.3.b.)}$$

$$\text{[nRBC]} = \text{measured [WBC]} \times \frac{\#\,\text{nRBC}/100\text{WBC}}{100 + \#\,\text{nRBC}/100\text{WBC}} \qquad \textbf{(2.3.c.)}$$

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

$$\text{[nRBC]} = 20{,}000/\mu\text{L} \times \frac{25}{125} = 4{,}000/\mu\text{L}$$

$$\text{[nRBC]} = \text{corrected [WBC]} \times \frac{\#\,\text{nRBC}}{100\ \text{WBC}} \qquad \textbf{(2.3.d.)}$$

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

$$\text{[nRBC]} = \frac{16{,}000\ \text{WBC}}{\mu\text{L}} \times \frac{25\ \text{nRBC}}{100\ \text{WBC}} = \frac{4{,}000\ \text{nRBC}}{\mu\ \text{L}} = 4{,}000/\mu\text{L}$$

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Table 2.3. Evaluation of reticulocyte values

Canine	Units	Reference intervals	Dog 1[a]	Dog 2[b]	Dog 3[c]
Hct	%	37–55	15	15	15
RP	%	0.0–1.5	3.0	9.0	0.0
CRP	%	0.0–1.5	1.0	3.0	0.0
[RBC]	× 10^6/μL	5.5–8.5	2.0	2.0	2.0
RC	× 10^3/μL	0–80	60	180	0

Feline	Units	Reference intervals	Cat 1[d]	Cat 2[e]	Cat 3[f]	Cat 4[g]	Cat 5[h]
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	20.0	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	6.7	10.0	20.0	2.0	0.3
[RBC]	× 10^6/μL	5.0–10.0	2.0	2.0	4.0	2.0	2.0
RC (aggregate)[i]	× 10^3/μL	0–40	120	40	24	24	2
RC (punctate)[i]	× 10^3/μL	50–300	400	600	1200	120	20

[a] Dog 1: Evidence of increased erythrocyte production is not present. If the marrow is currently responding to the anemia, it is not reflected in peripheral blood. The RP is increased because there are fewer erythrocytes, not because there are more reticulocytes in the blood.

[b] Dog 2: There is evidence of increased erythrocyte production; the marrow is currently responding to the anemia. The RP is increased because there are fewer erythrocytes and more reticulocytes in the blood than there are in health.

[c] Dog 3: There is not evidence of increased erythrocyte production. Because reticulocytes were not seen, decreased production of erythrocytes may be one reason for the anemia. Or, it could be too early for a reticulocyte response.

[d] Cat 1: There is evidence of increased erythrocyte production. The aggregate reticulocytosis indicates increased erythropoiesis in the past 3–6 days. The punctate reticulocytosis (↑ punctate RC) is consistent with the aggregate response and may persist for 1–3 weeks after initial stimulus.

[e] Cat 2: Because the release of punctate reticulocytes occurs sooner than the release of aggregate reticulocytes, the data could indicate the onset of anemia was within the past 2–3 days. If the anemia has been present longer, the aggregate CRP and RC values indicate a poor regenerative response. Possibly, the punctate reticulocytosis could be present because there was increased erythropoiesis 1–3 weeks ago.

[f] Cat 3: The magnitude of the punctate reticulocytosis indicates that a major stimulus for increased erythropoiesis occurred 1–3 weeks ago. Absence of an aggregate reticulocytosis suggests that the major stimulus has passed and erythroid hyperplasia is present in marrow.

[g] Cat 4: Evidence of increased erythrocyte production is not present; decreased production of erythrocytes is probably at least one reason for the anemia if there has been sufficient time for a marrow response.

[h] Cat 5: Evidence of increased erythrocyte production is not present. Decreased production of erythrocytes is probably contributing to the anemia because the reticulocyte concentrations are low normal to decreased.

[i] Reference intervals for absolute reticulocyte concentrations are estimates based on clinical experiences and published data for three cats. (Perkins PC, Grindem CB, Cullins LD. 1995. Flow cytometric analysis of punctate and aggregate reticulocyte responses in phlebotomized cats. Am J Vet Res 56:1564-1569)

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1. Basis of equation
   a. While you counted 100 leukocytes, you also counted 25 nRBC. Thus, you actually observed 100 leukocytes out of a total of 125 nucleated cells. Therefore, 100 of 125 nucleated cells (4/5) must be leukocytes.
   b. If the measured [WBC] was 20,000/µL, then 4/5 of the nucleated cells were leukocytes and thus the corrected [WBC] is 16,000 leukocytes/µL.
2. Calculation of the concentrations of the individual types of leukocytes is done after calculation of a corrected [WBC].

II. [nRBC]
   A. As mentioned above, the conventional method of reporting the enumeration of nRBC in blood is relative to 100 leukocytes. This method is useful for correcting a measured [WBC] but may give a false impression of the number of nRBC in blood. For example, two blood samples each have 50 nRBC/100 WBC. If one sample has a [WBC] of 50,000/µL and the second sample has a [WBC] of 500/µL, then the [nRBC] in first sample is 100 times the [nRBC] in the second sample.
   B. [nRBC] equations
      1. Eq. 2.3.b shows that the difference between a measured [WBC] and a corrected [WBC] is the [nRBC].
      2. Eq. 2.3.c shows the formula that is used to calculate a [nRBC] if the measured [WBC] and number of nRBC/100 WBC are known.
      3. Eq. 2.3.d shows the formula that is used to calculate a [nRBC] if the corrected [WBC] and number of nRBC/100 WBC are known.

COOMBS' TEST (DIRECT ANTIGLOBULIN TEST)

I. Purpose: To detect ESAIg or complement on a patient's erythrocytes, usually to help diagnose immune hemolytic anemia

II. Method for direct Coombs' test
   A. A patient's erythrocytes are washed 3 times with saline to remove nonbound proteins.
   B. Antiglobulin is added (species-specific anti-IgG, anti-IgM, or anti-complement). If sera contain two or more types of antiglobulin, then they are called polyvalent antisera. Antiglobulins will bind to any IgG, IgM or complement on the washed erythrocytes.
   C. A source of fresh complement proteins is needed for hemolytic reaction of the equine Coombs' test. The hemolytic reaction is needed because equine erythrocytes may not agglutinate in the direct Coombs' test.
   D. Positive reactions
      1. Agglutination indicates that a patient's erythrocytes are coated with hundreds of immunoglobulin or C3 molecules and there was sufficient binding of the immunoglobulin of antiserum to cause agglutination (Fig. 2.2).
         a. For agglutination to occur, there must be the appropriate ratio of antigen and antibodies. To reduce the possibility of false negative results due to the prozone response, Coombs' tests are frequently completed with multiple dilutions of antiglobulin sera.
         b. If there is a prozone reaction, the Coombs' test might be negative at a 1:2 antiserum dilution, weakly positive at a 1:4 dilution, and strongly positive at a 1:8 dilution.

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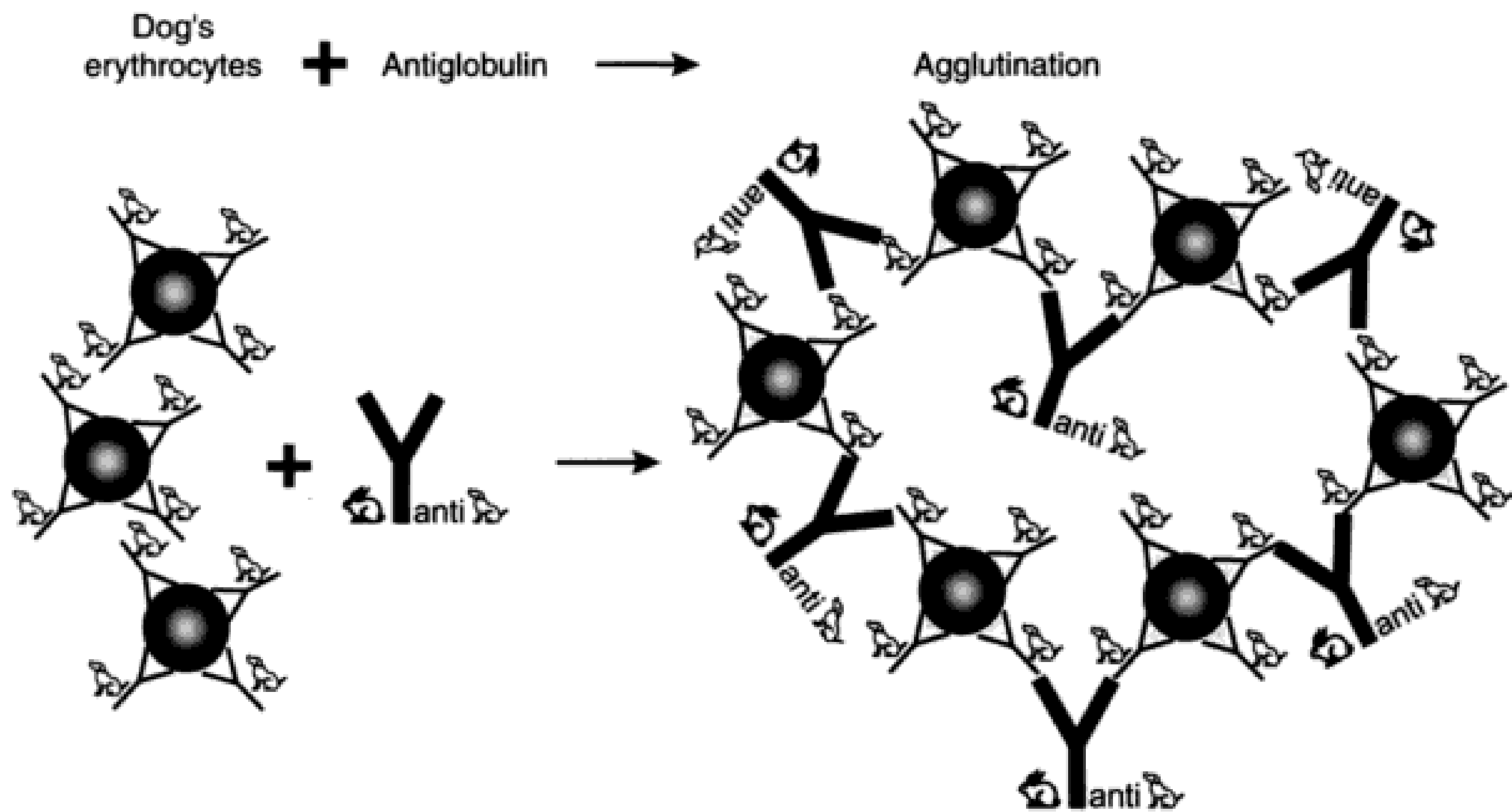

Fig. 2.2. Agglutination reaction of a positive canine Coombs' test. Washed canine erythrocytes are incubated with rabbit anti-dog immunoglobulin. If the dog's washed erythrocytes are coated with dog immunoglobulin, the antiserum will cause agglutination of the dog's erythrocytes when the concentration of rabbit anti-dog immunoglobulin is appropriate.

2. Hemolysis suggests that immune complexes formed between the antiserum immunoglobulin and the ESAIg, and these immune complexes led to complement activation and erythrocyte lysis.

III. Coombs' tests are not standardized and are often unnecessary given other clinical and laboratory findings. Negative results are common in cases that present, progress, and respond like immune hemolytic anemias; positive results do occur in samples from animals without clinically significant hemolysis.

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Chapter 3

LEUKOCYTES

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Table 3.1. Abbreviations and symbols in Chapter 3

»	symbol in tables to indicate relatively common disease or condition
ASVCP	American Society for Veterinary Clinical Pathology
BFU-E	burst forming unit–erythroid
BLV	bovine leukemia virus
C5a	complement, fragment 5a
CFU-Baso	colony forming unit–basophil
CFU-E	colony forming unit–erythroid
CFU-Eo	colony forming unit–eosinophil
CFU-G	colony forming unit–granulocyte
CFU-GM	colony forming unit–granulocyte/macrophage
CFU-M	colony forming unit–monocyte
CFU-Mast	colony forming unit–mast cell
CFU-Meg	colony forming unit–megakaryocyte
CLP	circulating lymphocyte pool
CNP	circulating neutrophil pool
FeLV	feline leukemia virus
f-MLP	N-formyl-methionyl-leucyl-phenylalanine
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
IgE	immunoglobulin E
IL-x	interleukins (x for Arabic numbers)
INFγ	interferon gamma
LTB_4	leukotriene B_4
MatNP	maturation neutrophil pool
MLP	marginated lymphocyte pool
MNP	marginated neutrophil pool
MPS	mucopolysaccharidosis
PAF	platelet activating factor
ProNP	proliferation neutrophil pool
SNP	storage neutrophil pool
TNFα	tumor necrosis factor alpha
TNFβ	tumor necrosis factor beta
TNP	tissue neutrophil pool
URL	upper reference limit
WBC	white blood cell (leukocyte)
WRI	within reference interval

PHYSIOLOGIC PROCESSES INVOLVING LEUKOCYTES

I. Leukon: all leukocytes in an animal, including leukocyte precursors, leukocytes in blood and lymph vessels, and tissue leukocytes
   A. Bone marrow contains precursors for neutrophils, eosinophils, basophils, monocytes, lymphocytes, and mast cells (considered tissue leukocytes).
      1. Leukopoiesis is part of hematopoiesis, which is a complex system involving stem cells that are capable of self-renewal or differentiation toward a committed cell line (Fig. 3.1).

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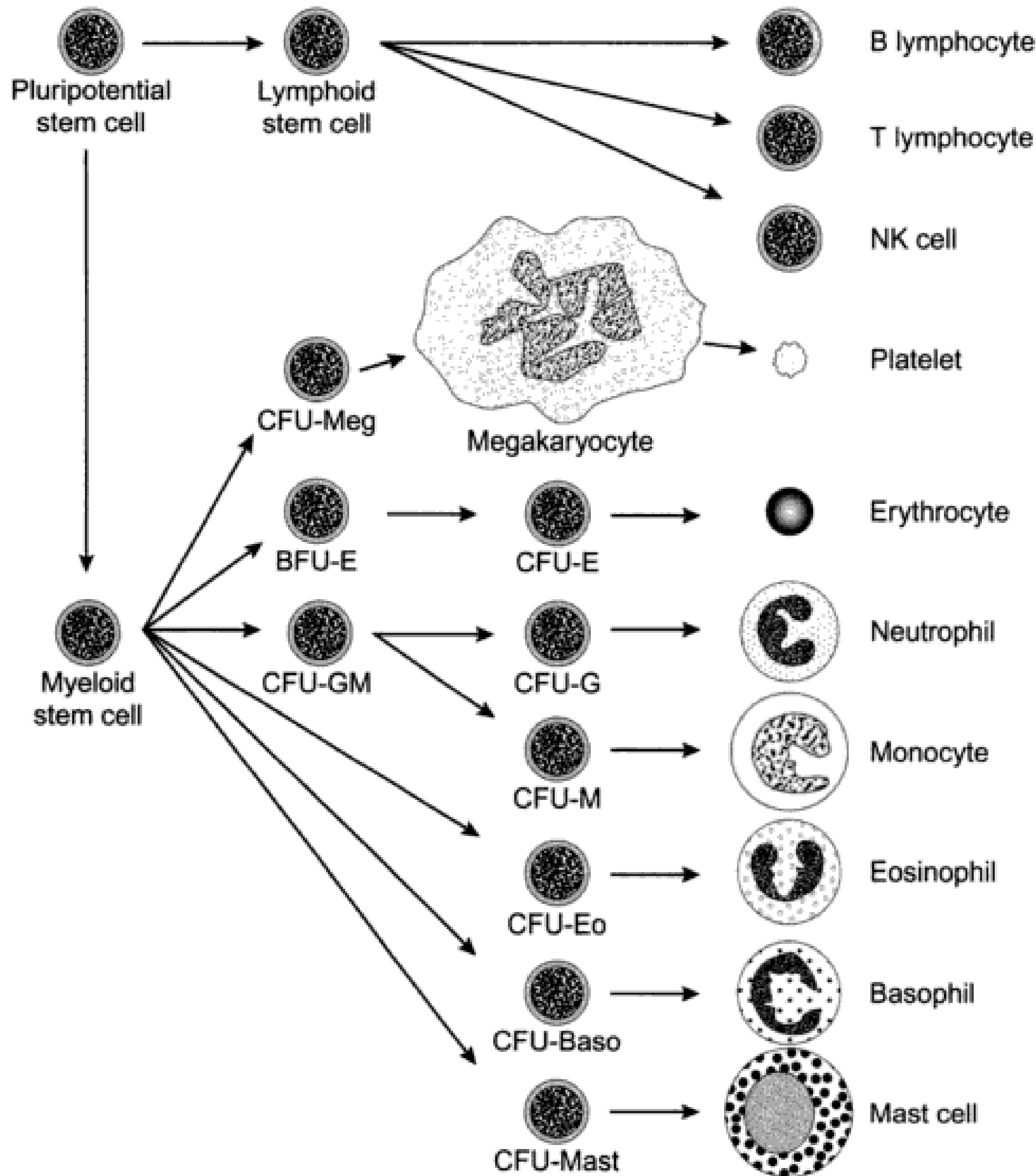

Fig. 3.1. Differentiation of pluripotential stem cells to the committed cell lines of the hematopoietic system. Major features of the hematopoietic system include the following: (1) Proliferation of myeloid and lymphoid cells classically occurs in marrow and other lymphoid tissues, respectively, and (2) there are seven nonlymphoid cell types and three major types of lymphoid cells produced by the system.

2. Stem cells look like small lymphocytes via light and transmission electron microscopy and are present in bone marrow, spleen, liver, and blood.
3. Specific stimuli and regulators govern leukocyte differentiation and production.

B. Lymph nodes, spleen, and thymus contain precursors for B, T, and null lymphocytes.

C. Leukocytes (white blood cells, WBC) in blood are in transit from sites of production to sites of function or destruction.

D. Tissue leukocytes

1. Granulocytes (neutrophils, eosinophils, and basophils) perform their roles in host defense and die.
2. Lymphocytes may undergo blastogenesis, return to blood via lymphatic vessels, or die.
3. Monocytes transform into histiocytes or macrophages that are capable of mitosis, perform their host defense functions, and die.
4. Mast cell precursors differentiate into tissue mast cells, perform their roles in host defense, and die; subsets are known.

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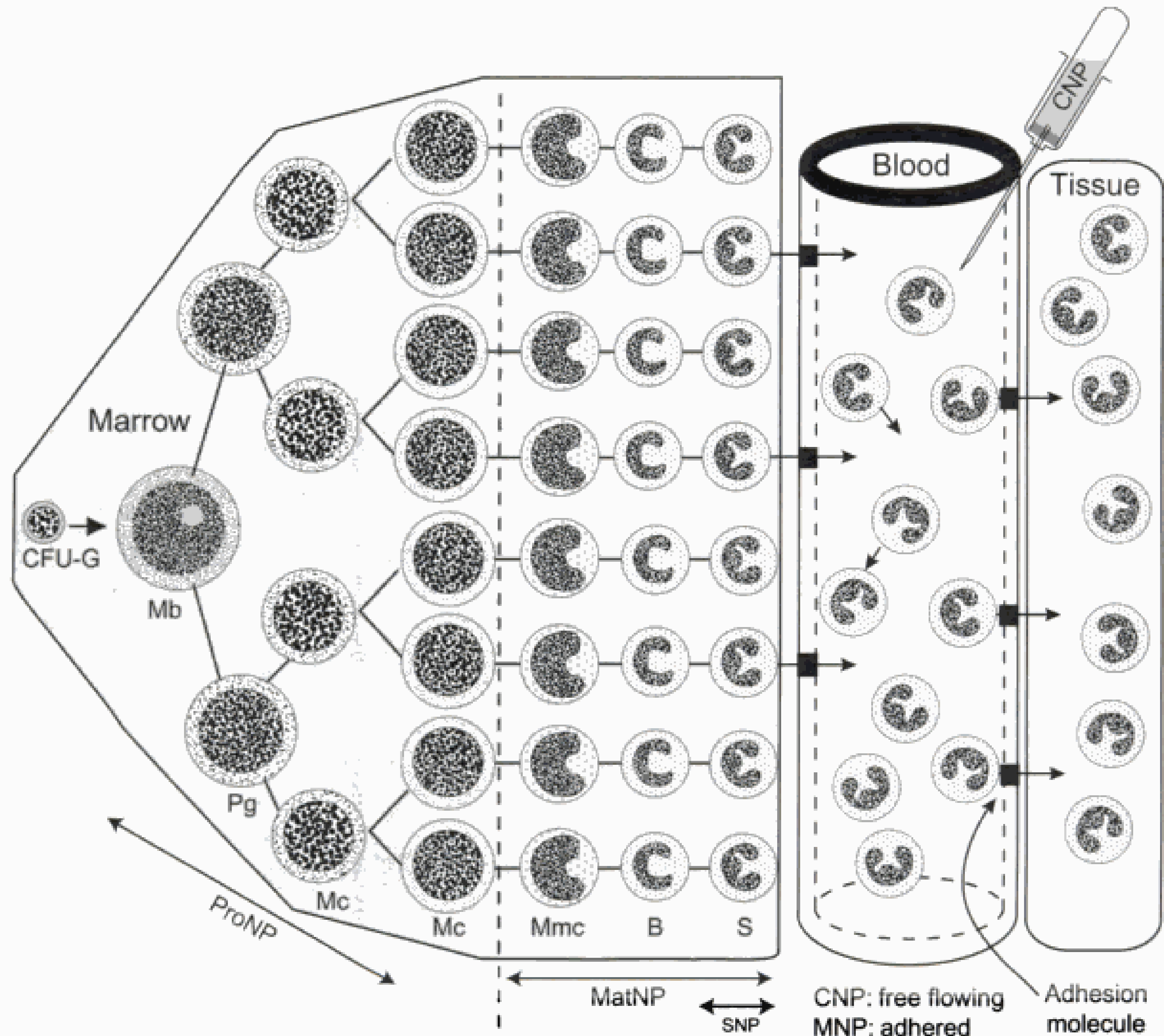

Fig. 3.2. Neutrophil kinetics in health.

Marrow has three major neutrophil pools: (1) self-renewal stem cells (CFU-G); (2) ProNP, or mitotic pool, that contains myeloblasts (Mb), progranulocytes (Pg), and myelocytes (Mc); and (3) MatNP, or postmitotic pool, that contains metamyelocytes (Mmc), band neutrophils (B), and segmented neutrophils (S). A SNP is within the MatNP and contains segmented neutrophils. When neutrophils leave the marrow and enter blood, they distribute between the MNP and CNP. After neutrophils bind to adhesion proteins on endothelial cells, they migrate into tissues to form the TNP. In the TNP, the neutrophils perform their protective functions and die.

II. Neutrophil pools and kinetics (movement of neutrophils) (Fig. 3.2)

A. Marrow neutrophils

1. IL-1, IL-3, IL-6, GM-CSF, and G-CSF stimulate CFU-G to differentiate into the neutrophilic cell line and to enter the ProNP (mitotic pool).
2. Myeloblasts, progranulocytes (promyelocytes), and myelocytes divide and mature in the ProNP. Programmed cell death occurs in the myelocyte stage to limit neutropoiesis in health. During enhanced neutropoiesis, fewer cells die in this stage and thus more enter the MatNP (postmitotic pool). In healthy mammals, neutrophil precursors are in this pool for about 3 days.
3. In the MatNP, 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 SNP (storage neutrophil pool). Neutrophilic cells are in the MatNP for 2-3 days in dogs and 4-6 days in people. The MatNP:ProNP ratio is about 4-6, meaning that the number of cells in the MatNP is 4-6 times the number of cells in the ProNP.

4. In health, segmented neutrophils are released from the MatNP to marrow sinuses and then to peripheral blood. Neutrophil releasing factors include chemoattractants (C5a, IL-8, f-MLP, LTB_4, PAF) and cytokine leukocytosis factors (IL-1, IL-6, TNFα, TNFβ, G-CSF, GM-CSF).

B. Blood neutrophils
   1. In health, neutrophils have a blood half-life of about 5-10 hr before they enter tissues.
   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 (circulating neutrophil pool).
      b. Neutrophils that temporarily adhere to endothelial cells are in the MNP (marginated neutrophil pool). The MNP is located primarily in small capillaries and veins in which neutrophils have the most opportunity to contact endothelial cells. After adhesion, neutrophils may break loose and re-enter the CNP or migrate into the TNP.
         (1) Inflammatory cytokines (including IL-1 and TNF from macrophages and INFγ from lymphocytes) stimulate endothelial cells to produce and express adhesion proteins (selectins) that mediate migration and "rolling."
         (2) Endogenous chemical mediators, including LTB_4 and PAF, activate neutrophils leading to expression of high affinity membrane integrins; these bind to endothelial cell receptors that mediate the process of migration into tissues.
      c. In most mammals, the MNP:CNP ratio is near 1. In cats, the ratio is 3.[1]
      d. Major processes that influence measured blood neutrophil concentrations
         (1) Production
            (a) Stem cell proliferation and differentiation
            (b) Effectiveness of maturation in myelocyte stage
         (2) Release from marrow: Oldest or most mature neutrophils preferentially leave marrow.
         (3) Distribution of neutrophils between the CNP and MNP
         (4) Migration from blood to tissues: Oldest or most mature neutrophils preferentially emigrate to tissue.

C. Tissue neutrophils
   1. Chemotactic substances such as C5a, IL-8, LTB_4, and PAF promote neutrophil migration to specific sites. Once in tissue, neutrophils do not return to blood.
   2. In health, most neutrophils die in respiratory and alimentary tissues.

III. Lymphocyte pools and kinetics (Fig. 3.3)

A. Lymph nodes and other lymphoid tissues
   1. Mediators stimulate differentiation and proliferation of B lymphocytes and T lymphocytes.
   2. Lymphocytes leave lymph nodes via efferent lymphatic vessels and enter blood via the thoracic duct.

B. Blood lymphocytes

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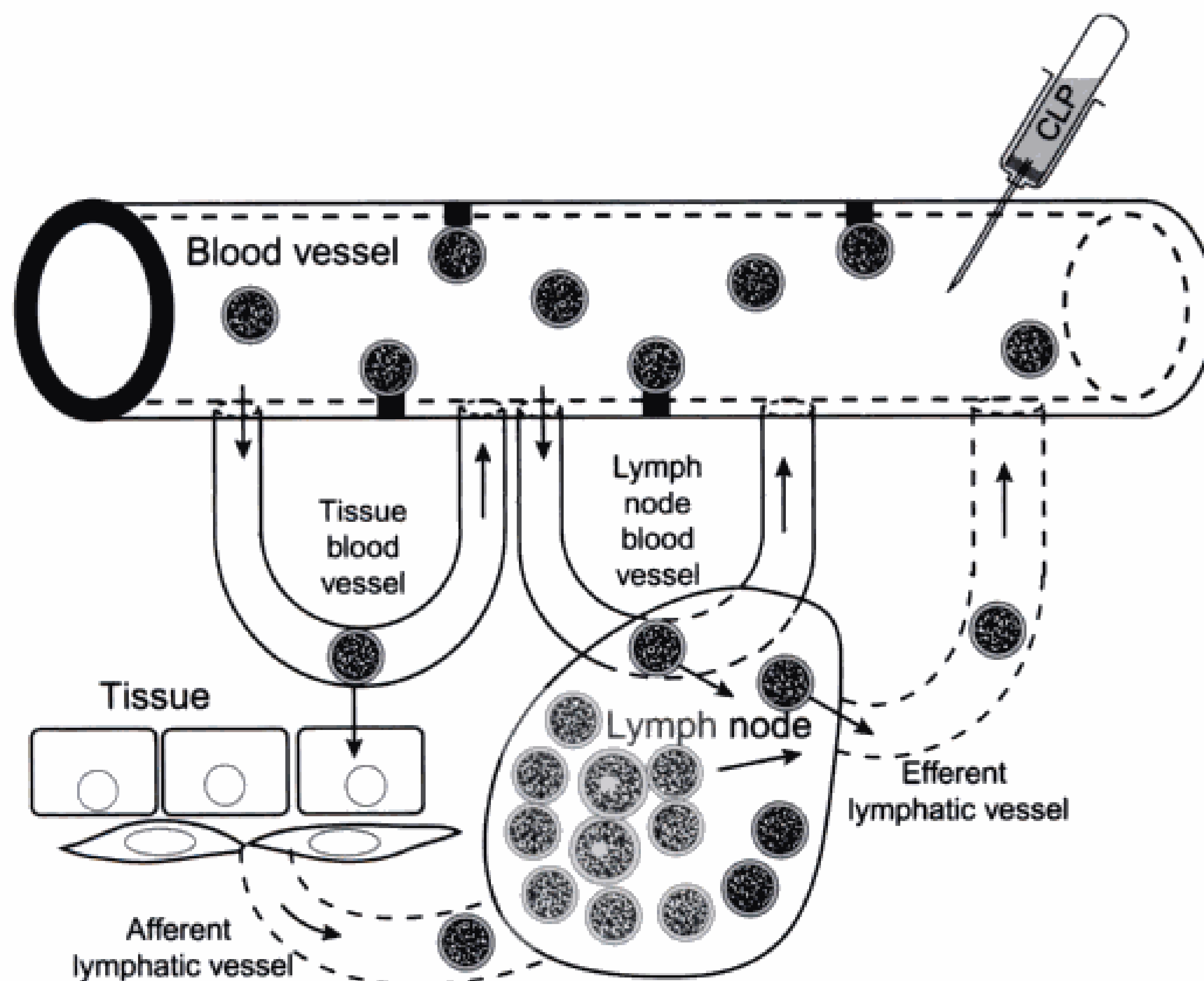

Fig. 3.3. Lymphocyte kinetics in health.
Lymph nodes and other primary lymphoid tissues are sites of lymphocyte production but also potential destinations of blood lymphocytes. Blood lymphocytes are distributed between marginal and circulating pools and may enter lymphoid organs or nonlymphoid tissues. Lymphocytes that enter nonlymphoid tissue may remain or may enter the afferent lymphatic vessels, be transported to regional lymph nodes, and then perhaps to blood via the thoracic duct.

1. Like neutrophils, lymphocytes are distributed into two pools: CLP and MLP. Most lymphocytes in blood are T lymphocytes.
2. From blood, lymphocytes enter lymph nodes and other tissues.

C. Lymphocytes in lymph nodes
  1. Lymphocytes migrate to lymph node cortices via specialized postcapillary venules (high endothelial venules); then they migrate through lymph nodes, exit via efferent lymphatic vessels, and return to blood.
  2. About 25% of blood lymphocytes enter lymph nodes each day through postcapillary venules, which have unique tall endothelial cells and receptors.

D. Lymphocytes in other tissues
  1. Lymphocytes emigrate to tissues to perform functions; there they may undergo blastogenesis, enter lymphatic vessels to return to blood, or die.
  2. Like neutrophils, migration to tissues involves lymphocyte chemotaxis and binding to endothelial cell receptors.

E. Major processes that influence measured blood lymphocyte concentrations
  1. Production
     a. Stem cell proliferation and differentiation
     b. Blastogenesis
  2. Distribution of lymphocytes between the CLP and MLP

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3. Migration from blood to lymph nodes and other tissues
4. Migration from lymph nodes via efferent lymphatic vessels

F. Lymphocyte life span varies from hours to years.

IV. Monocyte pools and kinetics

A. Monocytes and neutrophils share a common bipotential stem cell (CFU-GM) that is stimulated to differentiate by inflammatory cytokines.
B. When released from marrow, monocytes distribute between marginated and circulating pools.
C. Like other leukocytes, monocytes emigrate to tissues after binding to endothelial cells. Once in tissues, monocytes may differentiate into histiocytes, macrophages, or other specialized cells of the mononuclear phagocytic system.

V. Eosinophil pools and kinetics

A. Eosinopoiesis is stimulated by specific mediators including IL-5 (eosinophil differentiation factor) and GM-CSF from mast cells, macrophages, and lymphocytes.[2]
B. There are marginated and circulating blood eosinophils. They circulate in blood from minutes to hours and die in tissues (duration in tissues not established, maybe weeks or longer).
C. Eosinophils have phagocytic and bactericidal properties, they inactivate mediators from mast cells, and they attack larval and adult stages of a few parasites.

VI. Basophil pools and kinetics

A. Basophils originate in bone marrow, where their production and differentiation is controlled by IL-3 and other cytokines.
B. Their marrow transit time is at least 2.5 days, their circulating half-life is about 6 hr, and they may survive for as long as 2 weeks in tissues.
C. Basophil emigration to tissues is promoted by IL-1, TNFα, and endotoxin and is similar to the process used by neutrophils. Basophils are activated by IL-3 and IgE binding. Basophil granules contain substances that promote hypersensitivity inflammatory reactions and attract eosinophils.

VII. Mast cell kinetics

A. Mast cell precursors are found in marrow and are derived from a committed stem cell that is different from the basophil's committed stem cell.
B. Undifferentiated but committed mast cells leave marrow and circulate in blood but they are recognized only by special methods that are not used clinically. Finding differentiated mast cells in the blood of domestic mammals is considered a pathologic state.
C. Once in tissues, mast cell precursors differentiate into mast cells, undergo mitosis, or die. Mast cells play roles in hypersensitivity reactions, fibrosis, and other inflammatory responses in tissues. Mast cells are considered long-lived cells.

ABNORMAL LEUKOCYTE CONCENTRATIONS IN BLOOD

I. Abnormal neutrophil concentrations

A. Left shift

1. A left shift (shift to the left) is an increased concentration of nonsegmented neutrophils (usually bands) in blood. When there is great demand for neutrophils in

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tissues, younger stages (metamyelocytes, myelocytes, and rarely progranulocytes) may be present in the left shift.

a. Left shifts occur when release of neutrophils from marrow diminishes the SNP and younger cells are then released from the MatNP. The ability of neutrophils to respond to stimuli and migrate increases as they mature; thus segmented neutrophils respond first and immature forms respond later.
b. 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, neoplasia). Glucocorticoid hormones and endotoxins also stimulate release of neutrophils and thus may cause a mild left shift.

2. Left shift classifications
   a. Severity: Two different 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 the terms.
      (1) Immaturity of neutrophils in the left shift: bands (1^+ or slight); band and metamyelocytes (2^+ or moderate); band, metamyelocytes, and myelocytes (3^+ or marked)[3]
      (2) Magnitude of nonsegmented neutrophil concentrations: mild (< 1000/µL), moderate (1000-10,000/µL), marked (> 10,000/µL). (Ranges are provided as examples; such guidelines vary with species.)
   b. Regenerative versus degenerative left shift classifications
      (1) When first described by Dr. O.W. Schalm (father of veterinary hematology), specific criteria for classifying left shifts were not provided.[3]
         (a) A regenerative left shift is "characterized by a leukocytosis due to neutrophilia and with the appearance of immature neutrophils in peripheral blood."
         (b) In a degenerative left shift, "total leukocyte count remains within the normal range or is only slightly elevated, while the occurrence of young granulocytes in the circulation is prominent."
      (2) In the 1986 edition of *Schalm's Veterinary Hematology*, the states were described as follows:[4]
         (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."
         (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 leukocyte count is often suggestive of leukopenia, but it may sometimes be within the normal range or, rarely, elevated."
         (c) These descriptions allow for the classification of most left shifts. However, a neutropenia with mature forms exceeding immature forms does not fall within the classifications.
      (3) Clinical significance
         (a) As defined above, a regenerative left shift indicates that a neutrophil

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response to the inflammatory state is appropriate and at the time of sampling, neutrophil production and release are adequately responding to the demand.

(b) As defined above, a degenerative left shift suggests that neutrophil production and release are not adequately responding to the demand.

(4) Samplings from multiple days will better characterize the neutrophil response. If the regenerative left shift persists, then neutropoiesis is regenerating a replacement population of neutrophils that are being used in tissues to fight the invader. If the degenerative left shift persists, then there is inadequate neutropoiesis and the animal's condition is probably deteriorating (or degenerating).

B. A right shift (shift to the right) is an increased concentration of hypersegmented neutrophils in blood. Hypersegmented neutrophils have five or more nuclear lobes and usually 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 leukocyte differential count.

1. Glucocorticoid hormones (endogenous or exogenous) decrease emigration of neutrophils to tissue by down-regulating adhesion molecules, and thus can cause a right shift.
2. Other causes of a right shift: Poodle marrow dyscrasia syndrome,[5] FeLV-associated myelodyscrasia,[6], equine idiopathic hypersegmentation,[7] vitamin B_{12} deficiency in giant schnauzers with an inherited malabsorption syndrome,[8] occasionally chronic inflammatory diseases, and *in vitro* aging due to delayed analysis.

C. Neutrophilia (increased measured blood neutrophil concentration) (Table 3.2)

1. Acute inflammatory neutrophilia

Table 3.2. Diseases and conditions that cause neutrophilia

- Inflammatory
  - [a]Infections: bacterial, fungal, viral, protozoan
  - [a]Immune hemolytic anemia
  - [a]Necrosis: hemolysis, hemorrhage, infarcts, burns, neoplasia, sterile inflammation
  - Sterile foreign body
- Glucocorticoid-associated
  - [a]Stress (physical or neurogenic)
  - Hyperadrenocorticism
  - [a]Glucocorticoid therapy
  - ACTH therapy
- Physiologic (shift)
  - [a]Fight or flight response: excitement, fright, pain, exercise, anxiety
  - Catecholamine injections: epinephrine or norepinephrine
- Neoplastic
  - Granulocytic (myelogenous) leukemia
  - Paraneoplastic neutrophilia
- Others or unknown mechanisms
  - Neutrophilia of leukocyte adhesion deficiency
  - G-CSF administration
  - Estrogen toxicosis (early)

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a. This neutrophilia results from changes in neutrophil kinetics that are due to acute inflammatory mediators; the net result is an increased CNP that may contain a left shift (Fig. 3.4*B*).
   (1) Release from SNP occurs within hours after onset of inflammation and causes initial neutrophilia if release exceeds neutrophil emigration to inflamed tissue.
   (2) Release from MatNP causes a left shift and occurs after reduction or depletion of SNP.
   (3) Increased production from myelocyte stage: It takes 2-4 days before effects are seen in peripheral blood.
   (4) Increased production via stem cells: It takes about 5 days before effects are seen in peripheral blood; eventually will lead to granulocytic hyperplasia.

b. *Acute* refers to the type of inflammatory reaction and not the duration of the disease. The acute inflammatory pattern can be seen in an animal with a prolonged inflammatory state if there remains an active need for neutrophils in the inflamed tissue.

c. Inflammatory mediators must enter systemic blood and stimulate marrow cells for a neutrophilia to develop.
   (1) A neutrophilia is expected if there is substantial acute inflammation of subcutaneous tissues or 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 a neutrophilia because mediators are lost (to urine or skin) or do not leave the protected environment (brain or spinal cord).

d. The magnitude of inflammatory neutrophilias varies among species and thus interpretation of specific concentrations differs among species (Table 3.3).

e. The inflammatory bovine neutrophilia results primarily from increased production (granulocytic hyperplasia) and not from release of stored neutrophils because cattle have a small SNP.

f. The term *leukemoid response* has been used to describe any extreme inflammatory leukocytosis that is leukemia-like but proven not to be leukemic. The term can only be applied retrospectively. Disorders associated with a marked inflammatory neutrophilia include focal suppurative lesions (e.g., canine pyometra, pleuritis or pyothorax, peritonitis, prostatitis, pneumonia, abscesses), hemolytic anemia (especially immune-mediated) and canine hepatozoonoses.

g. When an animal has an acute inflammatory neutrophilia, it usually has a lymphopenia, often an eosinopenia, and occasionally a monocytosis, or toxic neutrophils. In dogs, a mastocytemia may be found.

Table 3.3. Species differences in the magnitude of inflammatory neutrophilia

	Reference intervals	Common magnitude	Occasional magnitude	Uncommon magnitude
Dogs	3,000–11,500[a]	12,000–30,000	30,000–60,000	> 60,000
Cats	2,500–12,500	13,000–25,000	25,000–40,000	> 40,000
Horses	2,250– 8,600	9,000–20,000	20,000–30,000	> 30,000
Cattle	600– 4,000	4,000–10,000	10,000–20,000	> 20,000

[a] Neutrophil concentrations expressed per μL of blood.

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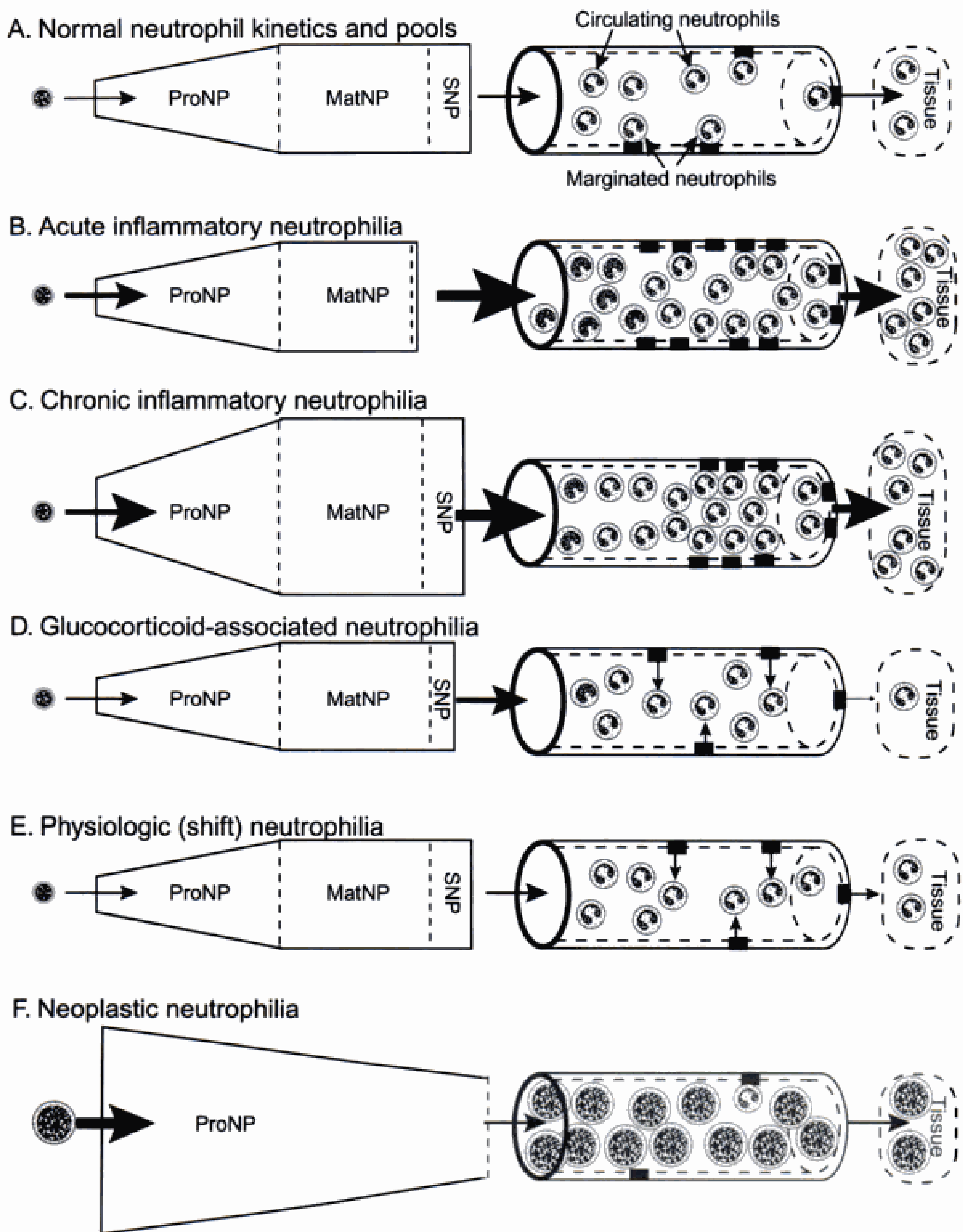

Fig. 3.4. Neutrophilia kinetics.

A. *Neutrophil kinetics in health* (reduced version of Fig. 3.2)

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 MatNP.

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 not be present because granulocytic hyperplasia maintains the SNP.

D. *Glucocorticoid-associated neutrophilia:* Neutrophilia occurs because of a shift of neutrophils from the MNP to the CNP, decreased migration of neutrophils to tissue, and release of neutrophils from the SNP and sometimes the MatNP.

E. *Physiologic (shift) neutrophilia:* Neutrophilia occurs because of the shift of neutrophils from the MNP to CNP.

F. *Neoplastic neutrophilia:* Neutrophilia occurs because of an uncontrolled proliferation of a clone of neoplastic neutrophil precursors. Acute myelogenous leukemia is depicted, but chronic myelogenous leukemia could also cause neutrophilia.

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2. Chronic inflammatory neutrophilia
   a. This neutrophilia results from changes in neutrophil kinetics when an inflammatory process continues at least a week during which inflammatory mediators stimulate development of granulocytic hyperplasia (Fig. 3.4*C*). If the SNP 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 due to granulocytic hyperplasia and increased neutrophil release from SNP 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, a neutrophilia develops.
   b. *Chronic* indirectly refers to the duration of the disease; the inflammatory process has persisted long enough to result in granulocytic hyperplasia.
   c. When an animal has a chronic inflammatory neutrophilia, other leukocyte abnormalities may include lymphocytosis (with or without reactive lymphocytes), monocytosis, eosinophilia, basophilia, a left shift, a right shift, and toxic neutrophils.
3. Glucocorticoid-associated (stress) neutrophilia
   a. This neutrophilia results from changes created by the effects of endogenous or exogenous glucocorticoids on neutrophil kinetics (Fig. 3.4*D*). Although this neutrophilia is frequently called a *stress neutrophilia,* it should not be confused with the physiologic (shift) neutrophilia that may result from the stress-induced release of catecholamines.
      (1) Neutrophils shift from the MNP to the CNP because the production of adhesion molecules is down-regulated.
         (a) This process can potentially double 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.
         (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.
      (2) Increased release of neutrophils from marrow: Mostly segmented neutrophils are released but glucocorticoids may cause release of band neutrophils and thus a mild left shift.
   b. Classic glucocorticoid leukograms are seen most frequently in dogs, where they consist of a mature neutrophilia (2-4× URL), lymphopenia, monocytosis, and eosinopenia. The magnitude of neutrophilia varies with different glucocorticoids and dosages. The effects on neutrophil kinetics diminish with chronic elevations in glucocorticoids.
   c. Blood may have a mild left shift (typically < 1000 band neutrophils/μL), a right shift, or no shift.
   d. Typical glucocorticoid effects differ among species of animals (Table 3.4).
4. Physiologic (shift) neutrophilia
   a. This neutrophilia results from effects of catecholamines (typically associated with fear, excitement, and exercise) that cause a shift from the MNP to the CNP (Fig.

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Table 3.4. Expected leukocyte concentrations in animals with glucocorticoid-associated leukograms

Leukocyte concentrations	Dogs	Cats	Horses	Cattle
Total WBC (/µL)	15,000–35,000	20,000–30,000	15,000–20,000	8,000–18,000
Segmented neutrophil	↑	↑	↑	WRI–↑
Band neutrophil	WRI–slight ↑	WRI	WRI	WRI
Lymphocyte	↓	↓–WRI[a]	↓–WRI[a]	↓
Monocyte	WRI–↑	WRI–↑	WRI	↓–WRI
Eosinophil	↓[b]	WRI[c]	WRI[c]	↓–WRI

[a] There is a paucity of species-specific data, but some references suggest that cats and horses may not develop glucocorticoid-induced lymphopenia as consistently as dogs and cattle.

[b] Decreased eosinophil concentrations in routine CBC results typically are not reliable because of the imprecision of leukocyte differential counts.

[c] Eosinopenia typically is not recognized because the lower reference limit for eosinophils is 0/µL.

3.4*E*). The shift may be due to the change in fluid dynamics that results from increased blood flow rate, especially in lungs.[9] Neutrophil adherence to endothelial cells may also be reduced.[10]

b. Magnitude of neutrophilia may be up to twice the URL for canine, equine, and bovine blood; potentially up to 3-4× URL for a cat because of a cat's larger MNP.

c. Seen most frequently in healthy animals and mostly in cats; leukocyte concentrations return to reference intervals relatively fast (hour) if the stimulus disappears.

d. Because the increased blood flow rate alters kinetics of other leukocytes, there also may be increased concentrations of other leukocytes, especially lymphocytes. The entire response results in a physiologic leukocytosis.

5. Neoplastic neutrophilia
   a. Neoplasia of neutrophilic cell line (granulocytic or myelogenous leukemia)
      (1) Neutrophilia is due to uncontrolled proliferation of cells. The causes of neutrophilic leukemias are generally not known. In cats, there is a high association with FeLV infections.
      (2) Typically, there is an extreme neutrophilia with a marked left shift, especially in bone marrow (Fig. 3.4*F*).
   b. Paraneoplastic leukocytosis
      (1) Neoplastic tissues produce G-CSF or a similar substance that stimulates neutropoiesis.
      (2) Canine neoplasms associated with extreme neutrophilic leukocytoses include rectal adenomatous polyp,[11] renal tubular carcinoma,[12] and metastatic fibrosarcoma.[13]
6. Other conditions or unknown
   a. Neutrophilia of leukocyte adhesion deficiency
      (1) Bovine leukocyte adhesion deficiency[14]
      (2) Leukocyte adhesion deficiency in Irish setter dogs[15]
   b. Neutrophilia induced by G-CSF administration
   c. Neutrophilia of estrogen toxicosis: Neutrophilia occurs 2-3 weeks after estradiol injections.[16]

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Table 3.5. Diseases and conditions that cause neutropenia

Inflammation
- Overwhelming tissue demand
  - ᵃOverwhelming bacterial infections: equine salmonellosis
  - ᵃSome viral infections: canine and feline parvovirus, equine influenza
  - ᵃVariety of inflammatory states in cattle: mastitis, pneumonia
- Endotoxemia of Gram-negative bacterial infections

Peripheral destruction
- Immune neutropenia
- Hemophagocytic syndromes

Granulocytic hypoplasia
- Infectious: parvovirus (dogs, cats), FeLV, *Toxoplasma, Ehrlichia*
- Neoplastic: primary or metastatic
- Toxic
  - Predictable: estrogen, chemotherapeutic drugs, chloramphenicol (cats), bracken fern
  - Idiosyncratic: phenylbutazone, griseofulvin
- Marrow necrosis
- Myelofibrosis

Ineffective production
- Immune neutropenia
- Suspected in animals: diphenylhydantoin and phenylbutazone toxicosis
- Chronic idiopathic neutropenia (G-CSF deficiency)

Cyclic hematopoiesis
- Cyclic hematopoiesis of gray collies
- Cyclic hematopoiesis associated with FeLV

Notes: Lists of specific disorders or conditions are not complete but are provided to give examples.
ᵃSome dogs of the Belgian Tervuren breed have lower neutrophil concentrations than reference intervals for most dogs. (Greenfield CL, Messick JB, Solter PF, Schaeffer DJ. 1999. Leukopenia in six healthy Belgian Tervuren. J Am Vet Med Assoc 215:1121-1122)

D. Neutropenia (decreased measured blood neutrophil concentration) (Table 3.5)
   1. Inflammatory neutropenia (overwhelming tissue demand) (Fig. 3.5*B*)
      a. Neutropenia occurs during an overwhelming or severe acute inflammatory disease. If associated with an endotoxemia, margination of neutrophils may be a dominant change in neutrophil kinetics.
      b. Neutropenia results when margination in vessels or emigration of neutrophils to inflamed tissue exceeds the release of neutrophils from marrow.
         (1) Cytokines and chemoattractants stimulate margination and emigration of large numbers of neutrophils to inflamed tissue. This movement may occur within hours of introduction of an infectious agent.
         (2) Neutrophil releasing factors stimulate a sudden release of neutrophils from the SNP. After the SNP is depleted, cells in the MatNP 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 MatNP because the total measured neutrophil concentration may be less than the URL for the band neutrophil concentration.
      c. Cytokines also stimulate increased production of neutrophils, but the results are not expected for at least 2 days.

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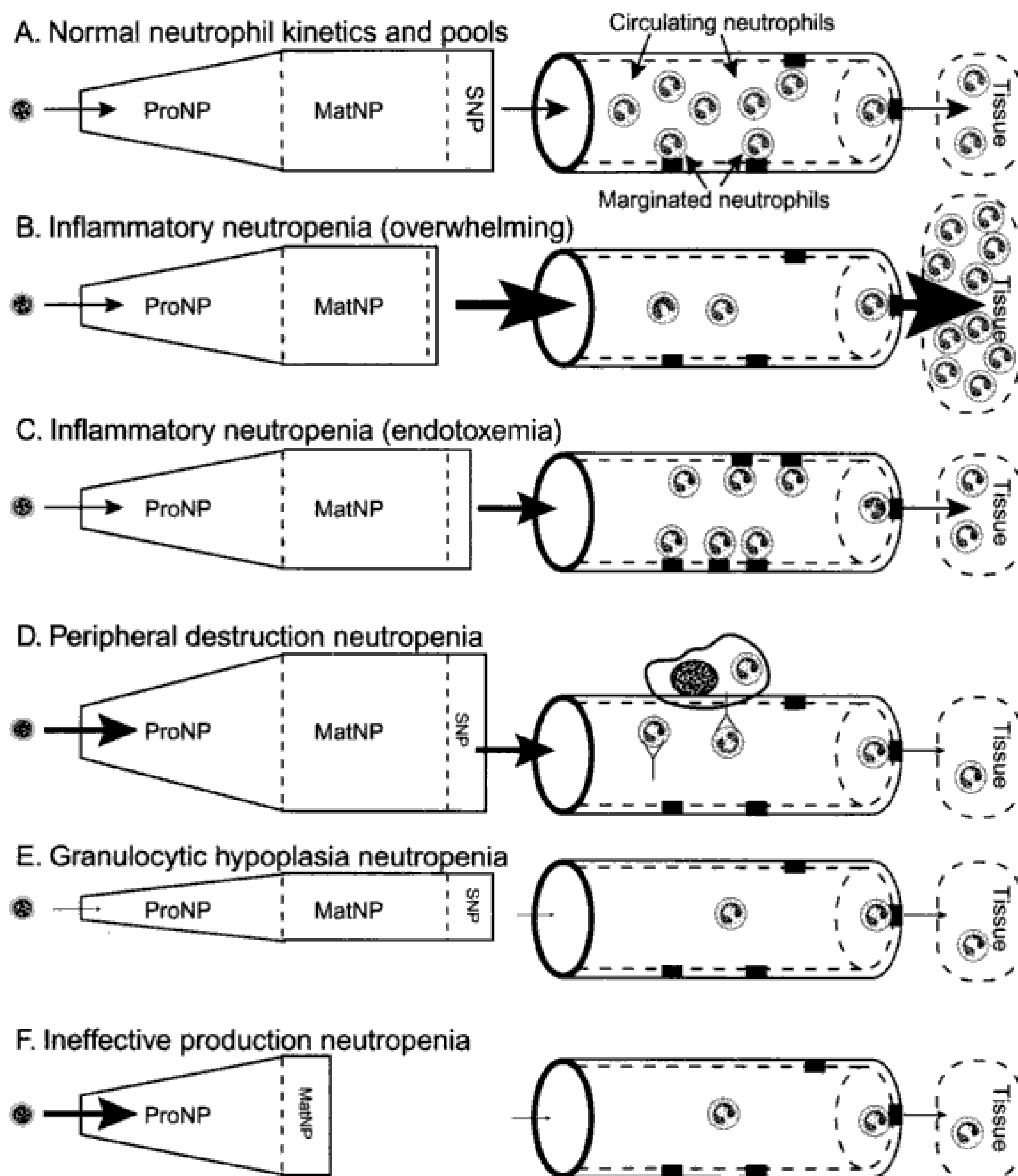

Fig. 3.5. Neutropenia kinetics.

A. *Neutrophil kinetics in health* (reduced version of Fig. 3.2)

B. *Inflammatory neutropenia* (overwhelming tissue demand): Neutropenia occurs because the margination and migration of neutrophils into the inflamed tissues exceeds the release of neutrophils from marrow.

C. *Inflammatory neutropenia* (endotoxemia): Neutropenia occurs because endotoxins stimulate the margination of neutrophils (sequestration of neutrophils in the MNP). Endotoxins may also affect marrow cells to cause increased release of neutrophils (see text).

D. *Peripheral destruction neutropenia:* Neutropenia occurs because neutrophils are being destroyed by macrophages; perhaps because of anti-neutrophil antibodies. If persistent, granulocytic hyperplasia will develop unless there is concurrent destruction of precursor cells.

E. *Granulocytic hypoplasia neutropenia:* Neutropenia occurs because there is decreased neutrophil production.

F. *Ineffective production neutropenia:* Neutropenia occurs because a disorder prevents an orderly maturation of neutrophil precursors in the marrow and thus there is decreased neutrophil production.

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d. If bone marrow is examined during the illness, cell populations reflect the changes in the leukon.
   (1) Early: depletion of SNP and possibly decreased MatNP
   (2) 2-3 days: increased ProNP without increased MatNP
   (3) 5-7 days: granulocytic hyperplasia from increases in both ProNP and MatNP
e. Inflammatory neutropenia is common in cattle because they have a relatively small SNP. Once there is a demand for neutrophils, there is not a sudden release of neutrophils from bone marrow to create a neutrophilia. The following sequence is the expected bovine response with acute inflammation induced by introduction of *Enterobacter aerogenes* into lactating quarters.[4]
   (1) Initial neutropenia (within 6 hr)
   (2) Significant left shift (by 24 hr)
   (3) Nadir of neutropenia (by 31 hr)
f. The severity of the neutropenia typically will be greater in bacteremic than non-bacteremic cattle with mastitis.[17]

2. Inflammatory neutropenia due to endotoxemia (Fig. 3.5*C*)
   a. This neutropenia occurs because endotoxins released from Gram-negative bacteria cause the rapid shift of neutrophils from the CNP to the MNP. This effect lasts for 1-3 hr after a single exposure. Endotoxins induce the release of inflammatory mediators (e.g., TNF, IL-1) that promote the adhesion of neutrophils to endothelial cells. Concurrent activation of the neutrophils may cause oxidative damage to endothelial cells.[18]
   b. Because of several factors, a neutropenia may no longer be present when a veterinarian examines an endotoxemic animal.
      (1) Endotoxins stimulate release of neutrophils from bone marrow in about 8-12 hr.
      (2) Endotoxins stimulate increased production of neutrophils, which affects blood neutrophil concentrations in about 3-5 days.
      (3) Other inflammatory mediators may concurrently alter neutrophil kinetics.
   c. Most studies of endotoxin-induced changes in neutrophil kinetics involved injection of endotoxins. In spontaneous infections, other mediators are probably also altering neutrophil movement.
3. Peripheral destruction neutropenia (Fig. 3.5*D*)
   a. Immune neutropenia[19,20]
      (1) Anti-neutrophil antibodies bind to neutrophils that are destroyed by the mononuclear phagocytic system.
      (2) Factors that induce the pathologic process are not established in domestic mammals.
      (3) Animals may be responsive to glucocorticoid therapy.
   b. Neutropenia of hemophagocytic syndrome[21-23]
      (1) Neutropenia may be one of multiple cytopenias associated with phagocyte hyperplasia.
      (2) In people, the acquired syndromes are associated with a variety of infectious and neoplastic states.[24]
4. Granulocytic hypoplasia neutropenia (decreased production neutropenia) (Fig. 3.5*E*)
   a. Granulocytic hypoplasia occurs when there is damage to either stem cells or cells of the marrow microenvironment. The hypoplasia causes decreased neutrophil production.

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b. This form of neutropenia is differentiated from other neutropenias by persistence of neutropenia (usually without a left shift, though secondary infections may result in left shifts) and bone marrow examination findings of granulocytic hypoplasia. Typically, maturation within the neutrophilic cell line is complete and orderly. Because the disease may cause damage to other cell lines in the marrow, concentrations of other blood cells (e.g., erythrocytes, platelets) may be decreased. There may not be a lymphopenia because lymphopoiesis in other lymphoid tissues may not be impaired.

5. Ineffective production neutropenia (neutropenia of ineffective neutropoiesis) (Fig. 3.5*F*)
   a. Ineffective production occurs when neutrophil precursors are defective or damaged and die before they are released from marrow.
   b. Lack of an orderly maturation sequence in marrow samples suggests there is a maturation arrest (e.g., hyperplasia up to one stage, then hypoplasia after than stage). There also may appear to be a failure to release (i.e., may have a persistent neutropenia and a concurrent granulocytic hyperplasia in the marrow).
   c. Ineffective neutropoiesis is characterized by a persistent neutropenia (with little or no left shift) concurrent with marrow hyperplasia prior to the defective stage. A monocytosis may be present because stimulation of CFU-GM results in more cells differentiating toward monocytes and neutrophils. Lymphocyte concentrations typically are WRI.
   d. Chronic idiopathic neutropenia was reported in a rottweiler dog that had a G-CSF deficiency.[25] The dog's marrow had granulocyte and mononuclear cell populations indicative of a maturation arrest. The findings suggested that G-CSF was needed for terminal neutrophil differentiation.
6. Cyclic hematopoiesis
   a. Canine cyclic hematopoiesis (cyclic neutropenia)[26]
      (1) Canine cyclic hematopoiesis is a hereditary disorder of gray collies and gray collie crosses. The specific defect is not known but involves either a pluripotent or totipotent stem cell.
      (2) Its diagnostic features result from 11- to 14-day cycles of neutrophil, erythrocyte, platelet, and monocyte production that result from cyclic differentiation of stem cells toward committed stem cells. The cycle of neutrophil production is out of sync with the other cycles.
      (3) Affected dogs are susceptible to infections because of recurrent neutropenia and are usually ill prior to 6 months of age. Anemia is usually mild because the life span of erythrocytes is longer than the 11- to 14-day cycle. A thrombocytosis may be present and defective platelet function has been described.
   b. Cyclic hematopoiesis associated with FeLV[27]

II. Abnormal lymphocyte concentrations
   A. Lymphocytosis (increased measured blood lymphocyte concentration) (Table 3.6)
      1. Chronic inflammatory lymphocytosis (Fig. 3.6*B*)
         a. This lymphocytosis results from increased lymphopoiesis in response to chronic antigenic or cytokine stimulation; reactive lymphocytes may be seen.
         b. Lymphocytosis is part of a hyperplastic lymphoid system; there may be concurrent enlarged lymph nodes or lymphoid hyperplasia in other tissues.

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Table 3.6. Diseases and conditions that cause lymphocytosis

Chronic inflammation
[a]Bacterial infections, especially rickettsial
Fungal infections, primarily systemic
Viral infections: FeLV, EIA, BLV
Protozoan infections, especially babesial and theilerial
Physiologic (shift)
[a]Fight or flight response: excitement, fright, pain, exercise, anxiety
Catecholamine injections: epinephrine or norepinephrine
Neoplasia
[a]Lymphoma (BLV, FeLV, idiopathic), leukemic phase
Lymphocytic leukemia
Hypoadrenocorticism

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Puppies, kittens, and foals have higher lymphocyte concentrations than mature animals of the respective species.

c. Lymphocytosis is usually mild to moderate (2-3× URL); occasionally there will be a marked lymphocytosis (> 30,000/µL).

d. Concurrent leukogram abnormalities commonly include a neutrophilia (mature, perhaps with right shift or left shift) and/or a monocytosis and occasionally an eosinophilia and/or basophilia.

2. Physiologic (shift) lymphocytosis (Fig. 3.6*C*)
   a. This lymphocytosis results from shifting of lymphocytes from the MLP to the CLP (especially from the spleen) that is promoted by exogenous or endogenous catecholamines. The lymphocyte shift is probably mediated through both increased blood flow rate and decreased lymphocyte adherence to endothelium.[10]
   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 but there are data to indicate that the catecholamine effect is mostly on NK cells, which may appear as large granular lymphocytes.[10]
   d. As with shift neutrophilia, shift lymphocytosis is seen most frequently in cats, young horses, and healthy animals.
3. Neoplastic lymphocytosis (Fig. 3.6*D*)
   a. This lymphocytosis results from 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 forms of lymphoid neoplasia in cats and cattle are caused by these viruses. Causes of lymphoid neoplasia in other animals are not known.
   c. BLV infections may induce a persistent lymphocytosis without the other features of lymphoid neoplasia (blast transformation, tissue manifestations of lymphoma).
   d. Usually the neoplastic lymphocytosis represents the leukemic manifestations of lymphoma; i.e., neoplasia started in lymph nodes (or other lymphoid tissues) and spread to blood. If neoplasia begins in bone marrow, then it is a primary lymphocytic leukemia.

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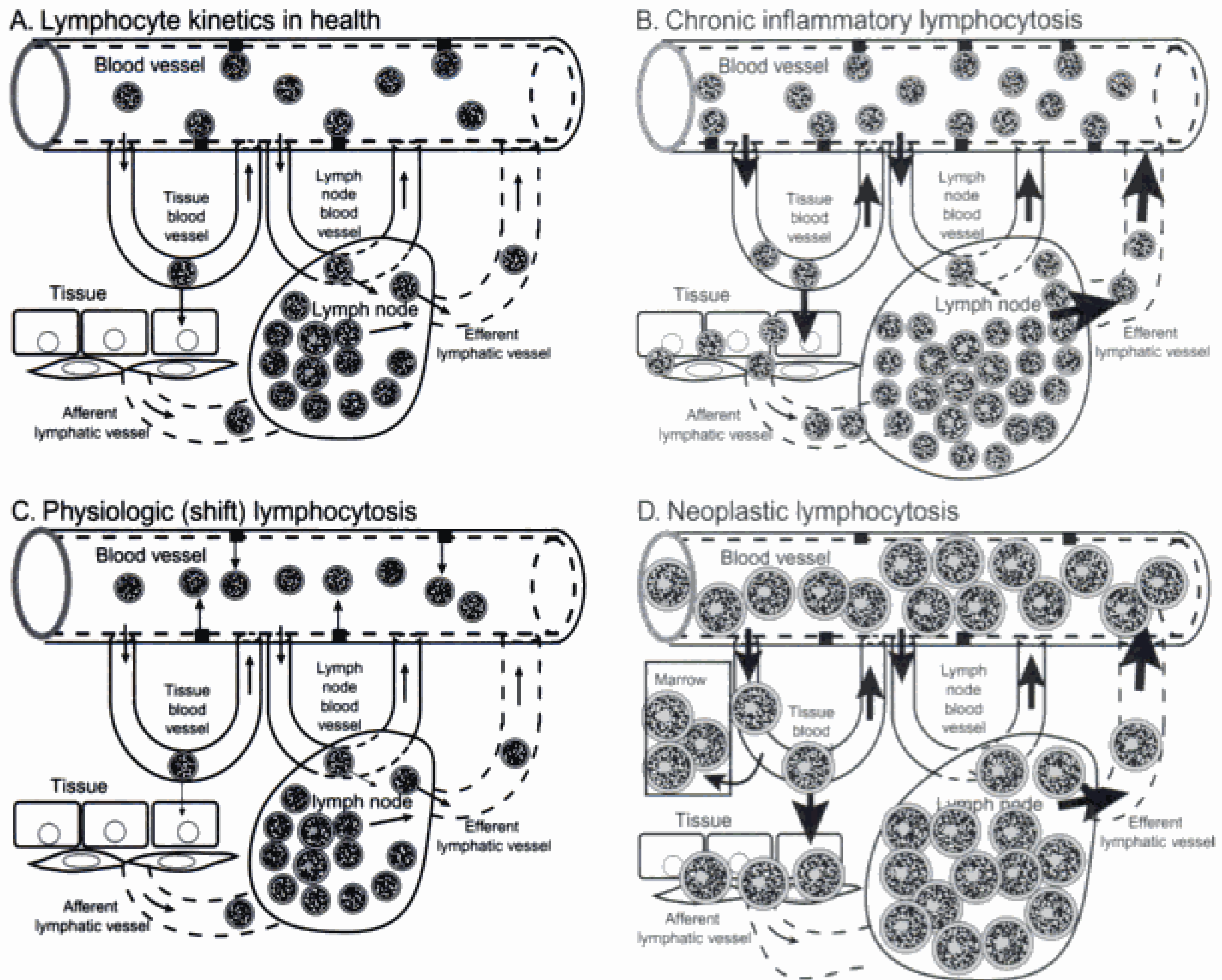

Fig. 3.6. Lymphocytosis kinetics.
A. *Lymphocyte kinetics in health* (reduced version of Fig. 3.3)
B. *Chronic inflammatory lymphocytosis:* Lymphocytosis occurs because of increased lymphopoiesis associated with the immune response to the inflammatory agent. The lymphocytosis is a part of lymphoid hyperplasia.
C. *Physiologic (shift) lymphocytosis:* Lymphocytosis occurs because of the shift of lymphocytes from the MLP to the CLP.
D. *Neoplastic lymphocytosis:* Neoplastic lymphocytosis occurs because of an uncontrolled proliferation of a lymphoid cell clone. This figure illustrates the leukemic manifestation of lymphoma; see text for other lymphoid leukemias.

e. Leukemia is classically characterized by a marked to extreme lymphocytosis with many lymphocytes having abnormal or "immature" morphologic features. The diagnosis is easiest if both criteria are present and difficult when they are not.

4. Lymphocytosis of hypoadrenocorticism (Addison's disease)
    a. This lymphocytosis may be due to an absence of glucocorticoid hormones, which normally inhibit lymphocyte production or alter lymphocyte distribution in the body.
    b. Dogs with hypoadrenocorticism may have a lymphocytosis. ALWAYS think of hypoadrenocorticism if you have an obviously stressed dog with a normal to decreased neutrophil concentration and lymphocytosis (stress should cause the opposite—neutrophilia and lymphopenia), especially in azotemic dogs.
    c. The classic leukogram of hypoadrenocorticism consists of a low-normal to decreased neutrophil concentration, high-normal to increased lymphocyte con-

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Table 3.7. Diseases and conditions that cause lymphopenia

- Acute inflammation
  - [a]Acute bacterial infections
  - [a]Acute viral infections
  - Endotoxemia
- Glucocorticoid associated
  - [a]Stress (physical or neurogenic)
  - Hyperadrenocorticism
  - [a]Glucocorticoid therapy
  - ACTH therapy
- Depletion
  - Lymphoid effusion: chylothorax, feline cardiomyopathy
  - Loss of afferent lymph: alimentary lymphoma, enteric neoplasms, granulomatous enteritis, paratuberculosis, protein-losing enteropathy, lymphangiectasia, ulcerative enteritis
- Lymphoid hypoplasia or aplasia
  - Immunosuppressive drugs or whole body irradiation
  - Destruction of lymphoid tissues: multicentric lymphoma, generalized lymphadenitis
  - Combined immunodeficiency of horses (Arabian, Appaloosa) and dogs (basset hound, Cardigan Welsh corgi, Jack Russell terrier)
  - Thymic aplasia of black-pied Danish cattle

Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

centration, a normal monocyte concentration, and a high-normal to increased eosinophil concentration.

5. "Lymphocytosis" of young animals
   a. Puppies, kittens, calves, and foals have higher blood lymphocyte concentrations than adult animals. Lymphocyte concentrations in cattle increase until about 1 yr of age and then gradually decrease over the years in adults.[4]
   b. Compared to adult reference intervals, the apparent lymphocytosis may be up to 2× URL.

B. Lymphopenia (decreased measured blood lymphocyte concentration) (Table 3.7)
   1. Acute inflammatory lymphopenia (Fig. 3.7*A*)
      a. This lymphopenia results from changes in lymphocyte kinetics stimulated by acute inflammatory mediators that reduce the CLP.[28]
         (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
         (3) Reducing the rate of lymphocytes leaving lymph nodes via efferent lymphatic vessels
      b. Most acute inflammatory leukograms with neutrophilia or neutropenia also have lymphopenia. Disappearance of lymphopenia is generally considered a good prognostic sign.
      c. Historically, lymphopenia was considered to be due to the stress associated with acute inflammation and not due to the inflammatory process itself. Stress of an illness may induce the lymphopenia, but documentation of such a pathogenesis was not found.

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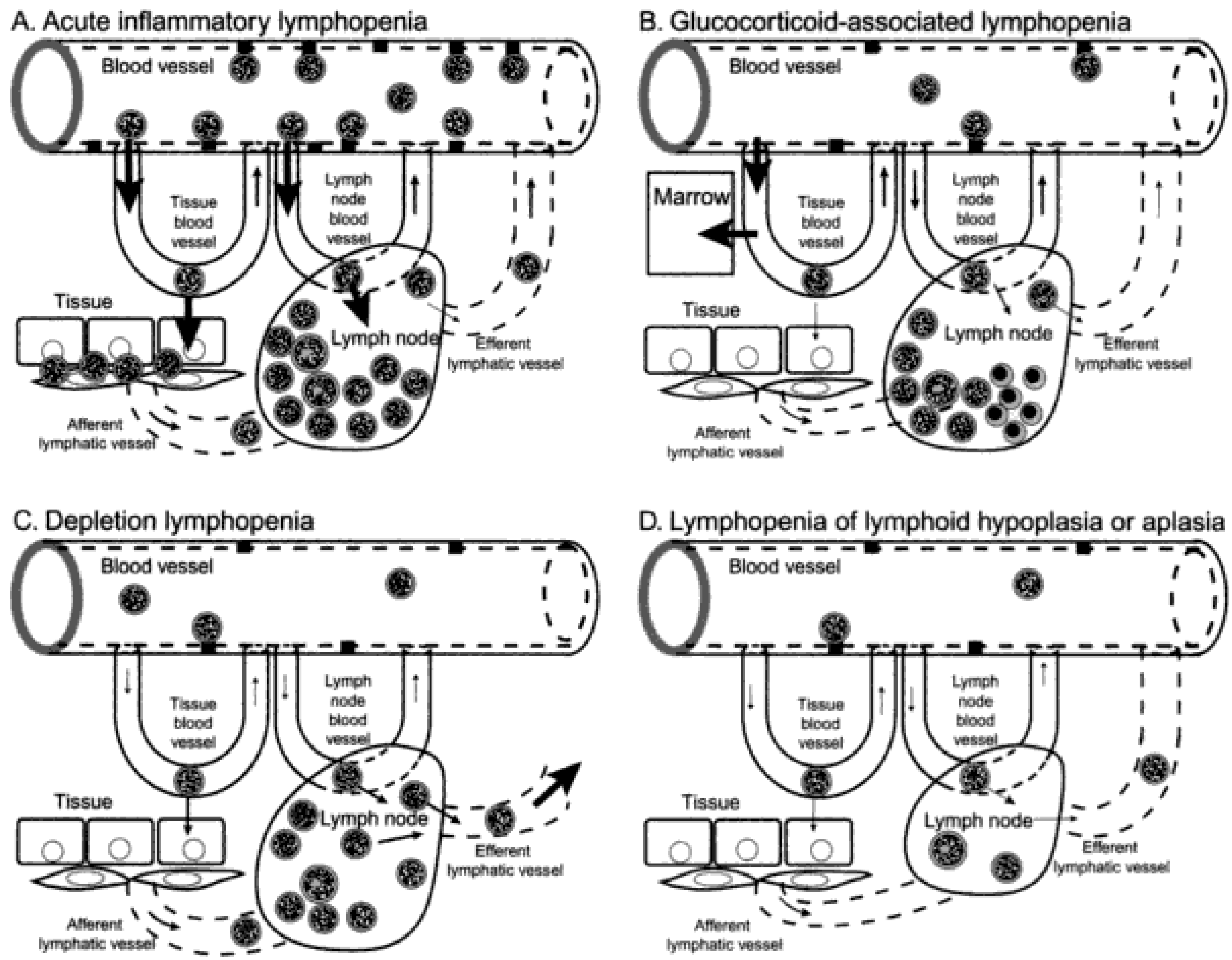

Fig. 3.7. Lymphopenia kinetics.
A. *Acute inflammatory lymphopenia:* Lymphopenia occurs because of (1) increased migration of lymphocytes to inflamed tissue, (2) homing of lymphocytes to lymphoid tissues, and (3) decreased movement of lymphocytes from lymph nodes back to blood.
B. *Glucocorticoid-associated lymphopenia:* Soon after administration of glucocorticoids, lymphopenia occurs because of the movement of lymphocytes to marrow or the decreased efflux of lymphocytes from lymph nodes. With persistent administration, glucocorticoids can become lymphotoxic, which leads to destruction of lymphocytes in lymph nodes and other tissues.
C. *Depletion lymphopenia:* Lymphopenia occurs because lymphocytes are lost from the vascular system with a loss of lymph or lymph-rich fluid.
D. *Lymphopenia of lymphoid hypoplasia or aplasia:* Lymphopenia occurs because of decreased lymphocyte production.

2. Glucocorticoid-associated (stress) lymphopenia (Fig. 3.7*B*)
   a. This lymphopenia results from the changes in lymphocyte kinetics caused by endogenous or exogenous glucocorticoids.
      (1) Immediate: shift of lymphocytes from CLP to other pools; once effects of glucocorticoids diminish, lymphocytes return to blood. Some reports indicate the lymphopenia is due to decreased efflux of lymphocytes from lymph nodes,[29] whereas other data indicate a redistribution to bone marrow.[30,31]
      (2) Later: Lymphotoxic effects cause lymphoid hypoplasia and thus decreased lymphopoiesis. Sensitivity of lymphocytes to glucocorticoids varies with the species and also with the stage of lymphocyte development.[32]

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Table 3.8. Diseases and conditions that cause monocytosis

Diseases and conditions
Inflammation
[a]Infections: bacterial (including rickettsial), fungal, protozoan
[a]Necrosis: hemolysis, hemorrhage, neoplasia, infarction, trauma
Glucocorticoid-associated
[a]Stress (physical or neurogenic)
Hyperadrenocorticism
[a]Glucocorticoid therapy
ACTH injection
Neoplasia: monocytic leukemia
Secondary to immune neutropenia
Cyclic hematopoiesis
G-CSF administration

Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

b. Typical causes are the same as mentioned for glucocorticoid-associated neutrophilia, and it is usually considered the most common lymphopenia in all species. The severity and duration of lymphopenia are generally proportional to dose and/or duration of increased glucocorticoids.

3. Depletion lymphopenia (Fig. 3.7*C*)
   a. This lymphopenia is produced by loss of lymphocytes from the body via loss of lymphocyte-rich lymph or an incomplete lymphocyte circulation pathway.
   b. Disorders that cause this lymphopenia are not common.
4. Lymphopenia of lymphoid hypoplasia or aplasia (Fig. 3.7*D*)
   a. This lymphopenia is caused by either congenital or acquired lymphoid hypoplasia or aplasia that results in decreased lymphocyte production.
   b. Because most blood lymphocytes are T lymphocytes, selective aplasia of T lymphocytes will cause a more severe lymphopenia than a selective aplasia of B lymphocytes.
5. Lymphopenia of lymphoma
   a. Lymphopenia is common in animals with lymphoma.
   b. The lymphopenia may be due to decreased production (damage to lymph nodes) or altered lymphocyte kinetics (disrupted circulation patterns).

III. Abnormal monocyte concentrations

A. Monocytosis (increased measured blood monocyte concentration) (Table 3.8)

1. Inflammatory monocytosis
   a. Acute and chronic inflammatory diseases may cause monocytosis by cytokine stimulation of monocyte production and release.
   b. Monocytosis generally reflects a need for macrophages in diseased tissue or blood.
2. Glucocorticoid-associated (stress) monocytosis
   a. Glucocorticoid hormones or drugs are a common cause of monocytosis in dogs and cats, but they cause minimal to no changes in horses and cattle.
   b. Monocytosis is probably due to a shift in monocytes from a marginated pool to a circulating pool.
3. Neoplastic monocytosis (monocytic leukemia)
   a. Typically characterized by a marked monocytosis with many morphologically abnormal monocytes

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   b. Relatively uncommon form of leukemia compared to granulocytic and lymphoid leukemias
4. Secondary to immune neutropenia: Because neutrophils and monocytes share a common bipotential stem cell, there may be increased monocytopoiesis when there is ineffective neutropoiesis.
5. Cyclic hematopoiesis (see Abnormal Leukocyte Concentrations in Blood, I.D.6)
6. G-CSF: G-CSF may be administered to promote neutropoiesis but it will also promote monocytopoiesis.

B. Monocytopenia: Monocytopenia is difficult to document because there can be relatively few blood monocytes in healthy domestic mammals. Monocytopenia is not considered a diagnostic problem.

IV. Abnormal eosinophil concentrations

A. Eosinophilia (increased measured blood eosinophil concentration)

1. Most eosinophilias appear to be related to eosinophil anti-inflammatory functions or to the attraction of eosinophils to tissues after mast cell or basophil degranulation.
2. An eosinophilia suggests the possibility of many disease states (Table 3.9).
3. Persistent mild eosinophilia is occasionally seen in clinically healthy mammals in which parasitism or other subclinical disease cannot be detected.
4. Many animals that have parasitic diseases (including heartworm disease) will not have an eosinophilia. Organisms that infect blood cells (e.g., *Haemobartonella, Babesia, Cytauxzoon, Hepatozoon*) are not expected to cause an eosinophilia.

Table 3.9. Diseases and conditions that cause eosinophilia

- Hypersensitivity (allergic) disorders
  - [a]Flea bite dermatitis
  - Hypersensitivity to staphylococcal or streptococcal proteins
  - Milk allergy in ruminants
  - Asthma and eosinophilic respiratory disorders
- Parasitism
  - Ectoparasites
  - [a]Heartworms
  - [a]Tissue nematodes, trematodes, and protozoa
    - Dogs: *Dirofilaria, Dipetalonema, Spirocerca, Strongyloides, Trichuris,* and *Paragonimus* infections; larval migration of hookworms and roundworms, *Habronema*
    - Cats: *Paragonimus, Aeleurostrongylus*
    - Horses: *Strongyloides*
    - Cattle: *Sarcocystis*
- Idiopathic eosinophilic conditions
  - [a]Dog: eosinophilic myositis, eosinophilic gastroenteritis, eosinophilic panosteitis, eosinophilic pneumonitis, eosinophilic granuloma complex in Siberian Huskies
  - [a]Cat: eosinophilic granuloma complex, eosinophilic enteritis, hypereosinophilic syndrome
- [a]Mast cell degranulation caused by inflammation: cutaneous, respiratory, intestinal, genital, urinary
- Mast cell neoplasia
- Hypoadrenocorticism
- Neoplastic eosinophilia (eosinophilic leukemia, paraneoplastic eosinophilia)

Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

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Table 3.10. Diseases and conditions that cause eosinopenia

Table 3.10
Glucocorticoid-associated
[a]Stress (physical or neurogenic)
Hyperadrenocorticism
[a]Glucocorticoid therapy
ACTH therapy
[a]Acute inflammation
Diseases causing a hypoplastic to aplastic marrow

Note: Low eosinophil concentrations in routine CBC results may not be reliable because of the imprecision of leukocyte differential counts.

B. Eosinopenia (decreased measured blood eosinophil concentration)
   1. By itself, eosinopenia is of little diagnostic significance. However, eosinopenia may be part of either an acute inflammatory or glucocorticoid leukogram (Table 3.10).
   2. Because eosinophil concentrations in healthy animals are typically low and a calculated eosinophil concentration in CBC results may not be accurate, the observed eosinopenia in CBC results may not truly represent a pathologic state. Also, reference intervals for eosinophil concentrations in some species have 0 as the lower reference limit.

V. Abnormal basophil concentrations
   A. Basophilia (increased measured blood basophil concentration)
      1. Only substantial or persistent mild increases in basophil concentrations above 200-300/μL should be considered definitive basophilias because of the imprecision of leukocyte differential counts (especially when dealing with a minority cell population).
      2. A cause of basophilia may not be apparent but can be associated with allergic, parasitic, and neoplastic states (Table 3.11).
   B. Basopenia (decreased blood basophil concentration): Basopenia cannot be documented with routine leukocyte differential counts because basophil concentrations are typically very low in domestic mammals. Basopenia is not known to be clinically significant.

Table 3.11. Diseases and conditions that cause basophilia

Table 3.11
Allergic reactions (immediate or delayed)
Drugs, foods, inhalants, insect stings/bites
Parasitism
Fleas
Gastrointestinal parasites such as nematodes
Vascular parasites such as *Dirofilaria immitis* and *Dipetalonema reconditum*
Neoplasia
Basophilic leukemia
Mast cell neoplasia
Feline myeloproliferative diseases
Lymphomatoid granulomatosis
Essential thrombocythemia
Polycythemia vera

Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

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Table 3.12. Disorders reported to be associated with mastocytemia

Neoplastic disorders
Cutaneous mast cell neoplasms
Visceral mast cell neoplasia
Non-neoplastic disorders in dogs
"Inflammatory
Enteritis, especially parvovirus
Fibrinous pericarditis and pleuritis
Bacterial peritonitis
Aspiration pneumonia
Acute pancreatic necrosis
Immune hemolytic anemias
Renal failure associated with acute inflammation
Inflammatory skin diseases: flea-bite hypersensitivity, atopy, sarcoptic mange, food allergy; some with secondary pyoderma
Hemorrhage secondary to hemophilia in dogs
Gastric torsion in dogs

VI. Abnormal mast cell concentrations
  A. Mastocytemia (mast cells detected in peripheral 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 non-neoplastic mast cell populations.
    2. Disorders that may cause a mastocytemia (Table 3.12)
      a. Mastocytosis in cats occurs in two forms, systemic and splenic, and mastocytemia may be seen in either.[33-37]
      b. Cutaneous mastocytoma is a common neoplasm of dogs that may spread to blood and hemic organs.[38]
      c. Non-neoplastic disorders in dogs (Table 3.12)[39-42]
  B. Because no mast cells are expected in blood of healthy domestic mammals, decreased concentrations do not occur.

LEUKOGRAM PATTERNS

I. Although each type of leukocyte is unique, alterations in blood leukocyte concentrations frequently occur in predictable patterns that are summarized in Table 3.13. 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 or "stress" pattern is probably most common in dogs.
  B. Cats
    1. Higher MNP:CNP ratios plus their "fight or flight" responses make them more prone to a physiologic leukocytosis.
    2. FeLV-induced changes create alterations in both cell concentrations and appearance.

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Table 3.13. Major leukogram patterns

Segmented neutrophil	Total WBC	Segmented neutrophil	Non-segmented neutrophil	Lymphocyte	Monocyte	Eosinophil
Acute inflammatory	↑	↑	↑	↓	WRI-↑	↓–WRI
Chronic inflammatory	↑	↑	WRI-↑	WRI-↑	WRI-↑	WRI-↑
Glucocorticoid associated	↑	↑	WRI-slight ↑	↓	↑	↓
Physiologic leukocytosis	↑	↑	WRI	↑	WRI-↑	WRI
Acute overwhelming inflammatory	↓	↓	WRI-↑	↓	WRI	↓–WRI
Acute inflammatory with endotoxemia	↓	↓	WRI	↓	WRI	↓–WRI
Granulocytic hypoplasia	↓	↓	WRI	WRI	WRI	WRI
Ineffective neutropoiesis	↓	↓	WRI	WRI	WRI-↑	WRI
Hypoadrenocorticism	WRI	↓–WRI	WRI	WRI-↑	WRI	WRI-↑
Hemic neoplasia	↑↑↑[a]	?	?	?	?	?

Note: See text for species differences, especially for cattle.

[a] Neoplastic cell line is expected to be increased; other cell lines typically are WRI or decreased.

C. Horses
   1. Horses frequently have little to no neutrophilia or left shift during inflammatory states.
   2. A pronounced left shift is uncommon unless toxic changes are present.

D. Cattle
   1. Common inflammatory states (such as mastitis or pneumonia) result in an inflammatory neutropenia because of the relatively small neutrophil storage pool.
   2. Inflammatory states can result in marked lymphocyte atypia that can be very difficult to differentiate from neoplastic changes.

II. Recognizing a leukogram pattern aids in the understanding of an animal's illness. However, not all disorders will produce classic patterns, concurrent processes can complicate patterns, and there is considerable overlap among some patterns.

ABNORMAL MORPHOLOGIC FEATURES OF LEUKOCYTES

I. Changes associated with inflammatory diseases
   A. Toxic neutrophil (see Plate 1.*A*, *B*, and *C*)
      1. Toxic neutrophils are neutrophils with any or all of the following characteristics that are called toxic changes.
         a. Foamy cytoplasm: cytoplasmic clearing due to dispersed organelles. (Note: Similar cytoplasmic appearance may be due to *in vitro* changes related to sample deterioration. The foamy vacuolization should not be confused with the clear, discrete vacuoles that develop in neutrophils exposed to EDTA for a few hours.)[43]

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   b. Diffuse cytoplasmic basophilia: retention or persistence of cytoplasmic RNA during maturation
   c. Cytoplasmic Döhle's inclusion bodies (focal cytoplasmic basophilia, Döhle bodies): irregular, round to angular, blue-gray cytoplasmic structures that are aggregates of rough endoplasmic reticulum that contains RNA
   d. Asynchronous nuclear maturation: Nuclei have finely granular lobes separated by filaments or pronounced indentations.
   e. Giant neutrophils: larger neutrophils released from marrow because of asynchronous cellular maturation
   f. Possibly hyalinized nuclei: severely damaged cell; may represent deterioration or autolysis
   g. Toxic granules: primary granules that are still visible in the later stages (usually not seen after the progranulocyte stage); uncommon in domestic mammals and seen mostly in horses
2. These structural changes in neutrophils represent maturation defects that occur during rapid neutropoiesis.
3. Apparently healthy cats may have neutrophils with Döhle's inclusion bodies. Therefore, feline Döhle's inclusion bodies without other toxic changes generally are not reported as a toxic change in cats unless they are frequent and prominent.
4 Neutrophils with toxic changes are commonly associated with severe bacterial infections but can be found in noninfectious states including after G-CSF administration, in turpentine-induced inflammation,[44] and in dyscrasias induced by cefonicid and cefazedone.[45]
5. Toxic changes can be graded based on types and degree of toxic changes, but classification systems have not been standardized. Probably the more severe the toxic changes, then the more severe the inflammatory state in the animal.
6. Toxic neutrophils (especially band and metamyelocyte stages) can be confused with some monocytes.
   a. If a cell is a toxic band, you should also be able to find similar toxic changes in segmented neutrophils.
   b. If a cell is a monocyte, you should be able to differentiate it from a segmented neutrophil that has the clear to pale pink cytoplasm.
   c. In some cases, immature toxic neutrophils cannot be reliably distinguished from monocytes on a Wright-stained blood film. In these cases, accuracy of a leukocyte differential count may not be as important as recognizing that left shifts and toxic changes indicate that animals have severe inflammatory diseases.

B. Giant neutrophils (see Plate 1.*C*)
   1. Giant neutrophils are segmented or band neutrophils with a diameter of 14-20 µm; they are larger than typical blood neutrophils with diameters of 11-13 µm. They probably form because of a skipped cell division in neutropoiesis.
   2. Giant neutrophils may be associated with increased neutropoiesis caused by inflammation, in which case they represent a toxic change; they are seen most commonly in cats.
   3. They also are seen in the Poodle Marrow Dyscrasia syndrome, FeLV infections, and after administration of chemotherapeutic drugs that inhibit cell division.

C. Hypersegmented neutrophils (see Plate 1.*D*)
   1. A hypersegmented neutrophil has a nucleus with five or more definitive lobes separated by filaments.

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2. They typically represent old neutrophils and are seen with increased blood concentrations of glucocorticoids (see Abnormal Leukocyte Concentrations in Blood, I.B). The aging effect can also be seen as an *in vitro* artifact of delayed sample analysis. Their cause is unknown in horses with idiopathic neutrophil hypersegmentation.
3. They may occur with myelodysplastic syndromes or leukemias involving the neutrophil series.

D. Reactive lymphocytes (see Plate 1.*E, F, G*, and *H*)
1. Reactive lymphocytes (plasmacytoid lymphocytes, immunocytes, virocytes) represent either stimulated T or B lymphocytes and can be found in blood of animals with a variety of acute and chronic inflammatory diseases. They are more common in chronic than acute inflammatory states. Rare reactive lymphocytes can be found in most blood samples but an increased number of them can indicate an inflammatory disease. They are common in young animals.
2. Reactive lymphocytes may have a variety of structural features that are considered to be reactive changes: increased amount of cytoplasm; enhanced cytoplasmic basophilia; perinuclear halo; prominent focal Golgi zone; and eccentric, enlarged, cleaved, convoluted, lobulated, or bilobed nuclei. The most consistent changes are increased amount of cytoplasm and increased cytoplasmic basophilia.
3. Reactive hyperplasia can result in lymphoblasts and other lymphoid cells with mitotic nuclei in the peripheral blood.
4. Individual reactive lymphocytes can be impossible to distinguish from neoplastic lymphoid cells, especially in cattle. The more pleomorphic the cells are, the more likely they represent neoplasia and not hyperplasia.

E. Changes in monocytes associated with inflammation (see Plate 1.*I*)
1. Monocytes can have increased cytoplasmic basophilia and hyperchromatic nuclei. Such "reactive monocytes" are seen in some inflammatory diseases, especially systemic fungal diseases and immune-mediated disorders. During microscopic examinations, they can be confused with typical and reactive lymphocytes.
2. Monocytes can also acquire the features of macrophages, with abundant gray to blue-gray cytoplasm, with or without vacuoles, and large round to oval nuclei. Cells with this appearance are usually associated with systemic infections such as histoplasmosis, ehrlichiosis, or leishmaniasis.

II. Leukocytes that contain hemosiderin, erythrocytes, or nuclear antigen-antibody complexes (LE cells)

A. Sideroleukocyte (see Plate 1.*J*)
1. A sideroleukocyte is a neutrophil or monocyte containing hemosiderin. On Wright-stained blood films, hemosiderin is a blue-green or yellow-brown pigment; its presence should be confirmed with an iron stain (Prussian blue, Mallory's).
2. Sideroleukocytes are rarely found but can be seen with hemolytic anemias (particularly equine infectious anemia) and posttransfusion.

B. Erythrophage (see Plate 1.*K*)
1. An erythrophage is a monocyte or neutrophil that has engulfed an erythrocyte.
2. It is occasionally seen in immune hemolytic anemias such as idiopathic immune hemolytic anemia in dogs, equine infectious anemia, and neonatal isoerythrolysis.

C. Lupus erythematosus (LE) cell
1. A LE cell is a neutrophil that engulfed nuclear antigen-antibody complexes. With a Wright stain, this material appears as pink or pale blue, homogenous inclusions of variable sizes.

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2. Rarely seen in domestic mammals even in the LE cell test that attempts to produce the cell *in vitro.*

III. Organisms in leukocytes
   A. Bacteria
      1. Rarely, bacteria may be found within neutrophils of bacteremic patients.
      2. When bacteria are found in blood, one must attempt to determine if the bacteria represent a bacteremia or a sample contaminated with bacteria.
   B. Canine distemper inclusions (see Plate 1.*L*)
      1. Inclusions appear as homogeneous, red to purplish red or pale blue, pleomorphic but homogeneous cytoplasmic inclusions; they are found in neutrophils, monocytes, lymphocytes, and erythrocytes. Quick stains (e.g., Diff-Quik) stain the viral particles better than traditional Romanowsky stains (e.g., Wright, Wright-Giemsa).
      2. They probably occur in the early viremic stage and before clinical illness. They are rarely observed in North America.
   C. Ehrlichial morulae (see Plate 1.*M, N,* and *O*)
      1. *Ehrlichia* spp. can invade and multiply in the blood leukocytes of many species (Table 3.14).
      2. Host range of the monocytic ehrlichial species tends to be limited, but the granulocytic forms are not as host specific.

Table 3.14. Major ehrlichial species that infect leukocytes of domestic mammals

Genogroup	Ehrlichial species	Disease name	Hosts (common listed first)	Cells infected	Frequency of finding morulae in blood leukocytes
I	*E. canis*	Canine ehrlichiosis, tropical pancytopenia	Dog	Monocyte, lymphocyte	Reported to be in 25%–30% of cases,[a] but are rare findings in more recently diagnosed cases
I	*E. ewingii*	Canine granulocytic ehrlichiosis	Dog People	Neutrophil, eosinophil, very rarely monocytes	0.1%–10% of neutrophils in first few days of illness; rare thereafter
II	*E. equi*	Equine ehrlichiosis	Horse Dog	Neutrophil, eosinophil	1%–50% of neutrophils in first few days of illness
II	*E. phagocytophila*	Tick fever, bovine ehrlichiosis	Cattle Sheep Goat	Neutrophil, eosinophil	7.5%–30% of leukocytes 5–10 days after infection[b]
III	*E. risticii*	Equine monocytic ehrlichiosis, Potomac horse fever	Horse Dog Cat	Monocyte	Very rare

Sources: [a]Troy GC, Vulgamott JC, Turnwald GH. 1980. Canine ehrlichiosis: A retrospective study of 30 naturally occurring cases. J Am Anim Hosp Assoc 16:181-187.

[b] Pusterla N, Huder J, Wolfensberger C, Braun U, Lutz H. 1977. Laboratory findings in cows after experimental infection with *Ehrlichia phagocytophila.* Clin Diagn Lab Immunol 4:643-647.

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3. Ehrlichial species that infect domestic mammals are usually considered separate from those that infect man (*Ehrlichia chaffeensis, E. sennetsu,* and human granulocytic agent). However, human isolates have been found that could not be distinguished from *E. ewingii* by nuclei acid sequencing and PCR testing.[46,47] Also, 16S rRNA and *groE* DNA sequences suggest that *E. equi, E. phagocytophila,* and the human granulocytic agent may be strains of the same species.[48,49]
4. *Ehrlichia* spp. are located in cytoplasmic vacuoles; the resulting clusters of organisms (2 to > 20) are called morulae, which have typical diameters of 1.5-4.0 µm and stain pale to dark blue-gray with a Wright stain. In granulocytic forms, there may be more than one morula per leukocyte. Morulae should not be confused with Döhle's inclusion bodies, platelets lying on leukocytes, or blebs of nuclear membrane.

D. *Hepatozoon americanum* (see Plate 1.*P*)
1. *H. americanum* is a protozoa whose gametocytes infect canine neutrophils and monocytes; prior to 1997, the North American organism was classified as *H. canis.*[50] Gametocytes are oval to elliptical, pale blue, and about 6-10 µm in length. 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.
2. In North America, it is found mostly in the southern or Gulf Coast states and may be associated with a leukocytosis.

E. *Histoplasma capsulatum* (see Plate 1.*Q*)
1. The yeast phase of *H. capsulatum* can be found singly or multiply in the cytoplasm of monocytes (or macrophages), neutrophils, and occasionally eosinophils of peripheral blood. Cells containing *Histoplasma* can usually be more easily found at the feathered edge of blood films (because larger cells are concentrated in the feathered edge) or on buffy coat preparations.
2. Organisms are round to oval, about 2-4 µm in length, with a prominent cell wall; internal structures are commonly eccentric and stain pale blue and/or dark pink to purple.
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.

F. *Leishmania* spp. (see Plate 1.*R*)
1. *Leishmania* is a protozoa that is found primarily in Mediterranean, Central American, and South American countries. However, it has been found in research colonies in Ohio and Oklahoma,[51,52] in Texas and Maryland dogs,[53,54] and in dogs from other southeastern states.[55]
2. Organisms (amastigotes) appear as oval to teardrop structures in macrophages; they are 2-3 µm long with eccentric reddish nuclei and small cytoplasmic red bars (kinetoplasts). Amastigotes must be differentiated from *Histoplasma,* which is about the same size, has a thicker cell wall, but does not have a kinetoplast.
3. Macrophages laden with amastigotes are usually numerous in bone marrow, lymph nodes, spleen, and liver.

G. *Mycobacterium* spp. (see Plate 1.*S* and *T*)
1. Rarely, a mycobacterial infection may become systemic and mycobacterial organisms will be found in peripheral blood neutrophils or monocytes.
2. Mycobacterial bacilli are unique in that they do not stain with a Wright stain (negative staining reaction) and thus appear as clear rods within neutrophils or macrophages.

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H. *Sarcocystis* spp.
  1. Large oval merozoites of *Sarcocystis* can be found in bovine mononuclear cells during experimental infections.[56,57] Merozoites are about 3-4 μm × 10-12 μm and contain azurophilic nuclear material.
  2. The hematogenous merozoite is not a reported observation during natural infections.

I. *Toxoplasma gondii* (see Plate 1.*U*)
  1. Tachyzoites of *Toxoplasma gondii* are rarely found in blood neutrophils and monocytes of dogs and cats with acute toxoplasmosis.
  2. They are more common in macrophages of infected tissues (lung, intestine).

IV. Hereditary disorders that have leukocyte inclusions. (All are rare.)

A. Chediak-Higashi syndrome[58]
  1. Chediak-Higashi syndrome is a hereditary disorder of blue-smoke Persian cats, Hereford cattle, other nondomestic mammals, and man.
  2. Diagnostic features include large specific granules in cytoplasms of neutrophils, eosinophils, and basophils. Abnormal granules reflect fusion of granules (lysosomes).

B. GM_1 gangliosidosis[59]
  1. GM_1 gangliosidosis, a hereditary disorder of Friesian cattle, Siamese and Korat cats, English springer spaniels, mixed breed beagle dogs, and Portuguese water dogs, is caused by a deficiency of β-galactosidase.
  2. Diagnostic features include small, distinct, clear cytoplasmic vacuoles in lymphocytes in Wright-stained blood films.

C. GM_2 gangliosidosis[60,61]
  1. GM_2 gangliosidosis is a hereditary disorder of Yorkshire pigs, German shorthaired pointers, and cats caused by a deficiency of β-hexosaminidase.
  2. Diagnostic features include dark blue granules in neutrophils and prominent azurophilic granulation in lymphocytes.

D. Hereditary anomaly of neutrophil granulation in Birman cats[62]
  1. This anomaly is an autosomal recessive trait in purebred Birman cats.
  2. Diagnostic features include prominent fine eosinophilic granulation in the cytoplasms of neutrophils that must be differentiated from toxic granules and inclusions of MPS Type VI. Ultrastructural morphologic features of granules are normal.

E. MPS Type I[63,64]
  1. MPS Type I is a hereditary disorder in domestic cats, dogs, and man caused by a deficiency of α-L-iduronidase.
  2. In some studies, leukocytic cytoplasmic inclusions were seen via transmission electron microscopy but not in routine light microscopy. Others have found that feline neutrophils have abnormal cytoplasmic granulation (small pink granules).

F. MPS Type VI[65]
  1. MPS Type VI is a hereditary disorder in Siamese cats, domestic shorthair cats, and dachshunds caused by a deficiency of arylsulfatase B.
  2. Diagnostic features include the presence of large reddish purple granules in neutrophils that should not be confused with toxic granules (toxic neutrophils have other toxic changes). Cytoplasmic inclusions (granules) represent an accumulation of mucopolysaccharide.

G. MPS Type VII[66,67]
  1. MPS Type VII is a hereditary disorder caused by a deficiency of β-glucuronidase.

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2. Neutrophils of dogs and cats with MPS Type VII have inclusions like those found in MPS Type VI.

H. Fucosidosis[68]

1. Fucosidosis is caused by a deficiency of α-L-fucosidase, an enzyme in glycoprotein metabolism.
2. Lymphocytes of dogs with fucosidosis have cytoplasmic vacuoles.

OTHER NON-NEOPLASTIC LEUKOCYTE DISORDERS

I. Bone marrow dyscrasia of poodles[69]

A. This bone marrow dyscrasia is a hereditary disorder seen in miniature and toy poodles. The developmental defect in cell production is not known and there is not a clinical problem associated with the syndrome.

B. Diagnostic features of the syndrome

1. Hypersegmented or giant neutrophils
2. Macrocytic normochromic erythrocytes without anemia or reticulocytosis; (1) MCV = 80-110 fL (most unaffected dogs: 60-77 fL); (2) MCHC values are WRI; (3) MCH values are increased (compared to most unaffected dogs).

II. Idiopathic hypersegmented neutrophils of a horse[7]

A. The diagnostic feature of this syndrome was hypersegmented neutrophils. Neutrophil nuclei had 3-11 definitive lobes separated by filaments and about 70% of nuclei had 7-10 lobes.

B. The hypersegmentation was not associated with the common causes of hypersegmentation, i.e., hyperadrenocorticism (Cushing's disease), exogenous glucocorticoids, or chronic suppurative inflammation. The cause of the syndrome was not determined; the possibility that hypersegmentation was due to B_{12} or folate deficiency was not excluded. Clinical significance was not known.

III. Pelger-Huët anomaly[70-72] (see Plate 1.*V*, *W*, and *X*)

A. Pelger-Huët anomaly is a hereditary disorder that occurs in several breeds of dogs and at least two cat litters (domestic shorthair). Most clinical cases involve heterozygotes for the anomaly; a homozygotic kitten was stillborn.[71]

B. The disorder is characterized by hyposegmentation of neutrophil, eosinophil, and basophil nuclei; the nuclei are round, oval, dumbbell shaped, or occasionally have partial segmentation; nuclear chromatin may appear hyperchromatic or normochromatic, but it lacks the paler appearance of typical band neutrophils. Monocyte nuclei have decreased lobulation.

C. Pelger-Huët neutrophils should not be classified as band neutrophils (or younger forms) because such classifications will result in a left shift and thus indicate an inflammatory disease.

IV. Pseudo-Pelger-Huët neutrophils[73,74]

A. Pseudo-Pelger-Huët neutrophils are seen in cows, occasionally in dogs with severe inflammation, and in cats with FeLV-induced myeloid leukemia.

B. The transient defect represents asynchronous neutrophil maturation; some neutrophils look like Pelger-Huët cells but others do not. Usually other toxic changes are present with severe inflammation.

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COLOR PLATES

Hematologic, Urinary, and Serum Protein Electrophoretic Abnormalities

1. Photomicrographs of leukocyte abnormalities.
2. Photomicrographs of erythrocyte abnormalities.
3. Photomicrographs of erythrocyte abnormalities.
4. Photomicrographs of platelet abnormalities.
5. Serum protein electrophoresis densitometer tracings, cellulose acetate strips, and serum protein concentrations from dogs and cats.
6. Photograph of urine and photomicrographs of urine sediment findings.

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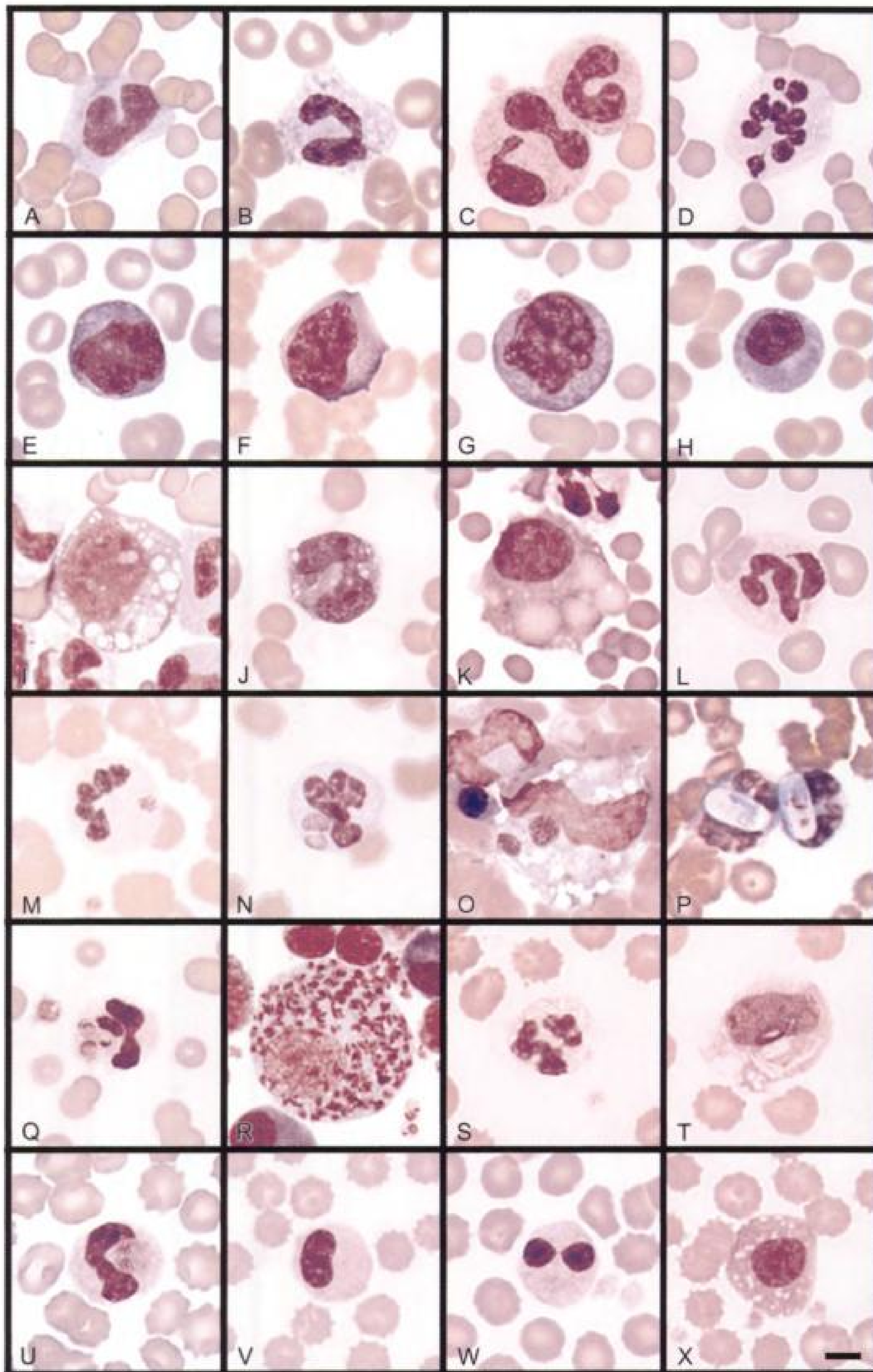

Plate 1. Photomicrographs of leukocyte abnormalities. (All are from Wright-stained blood films.)

A. Toxic band neutrophil with foamy cytoplasm that contains Döhle bodies, horse. *B.* Toxic neutrophil, dog. *C.* Toxic giant neutrophil with double nucleus and toxic band neutrophil, cat. *D.* Hypersegmented neutrophil, horse. *E.* Reactive lymphocyte, dog. *F.* Reactive lymphocyte, dog. *G.* Reactive lymphocyte, horse. *H.* Plasmacytoid cell, cat. *I.* Activated monocyte or macrophage, cat. *J.* Sideroleukocyte, dog. *K.* Erythrophage, horse. *L.* Distemper inclusion, dog (from ASVCP slide contributed by Dr. J.C. Tobey, 1993). *M.* Morula of *Ehrlichia ewingii* in a neutrophil, dog. *N.* Morulae of *Ehrlichia equi* in a neutrophil, horse (from ASVCP slide contributed by Dr. J.W. Harvey, 1983). *O.* Morulae of *Ehrlichia canis* in a macrophage of cerebrospinal fluid, dog. *P.* Gametocytes of *Hepatozoon americanum* in neutrophils, dog (from slide provided by Dr. R.A. Green). *Q.* Yeast stages of *Histoplasma capsulatum* in a neutrophil, cat. *R.* Amastigotes of *Leishmania* sp. in a macrophage, dog (from ASVCP slide contributed by Dr. K.S. Latimer, 1990). *S.* Negative-staining *Mycobacterium* sp. in a neutrophil, dog (from ASVCP slide contributed by Dr. H.W. Tvedten, 1988). *T.* Negative-staining *Mycobacterium* sp. in a monocyte, dog (from ASVCP slide contributed by Dr. H.W. Tvedten, 1988). *U.* Tachyzoites of *Toxoplasma gondii* in a neutrophil, dog. *V.* Pelger-Huët neutrophil, dog. W. Dumbbell form of Pelger-Huët neutrophil, dog. *X.* Pelger-Huët eosinophil, dog (bar = 5μm).

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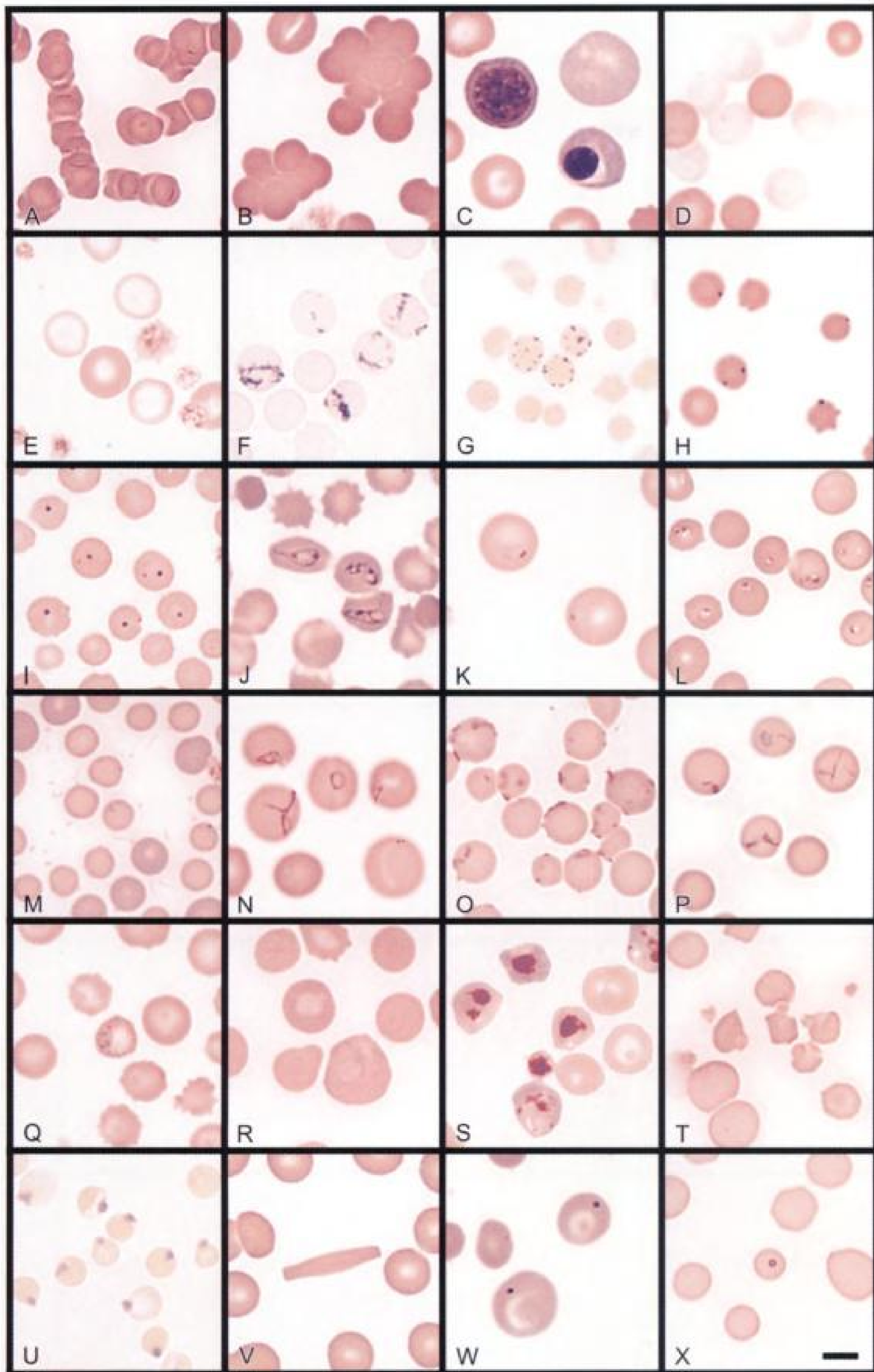

Plate 2. Photomicrographs of erythrocyte abnormalities. (All are from Wright-stained blood films unless otherwise stated)

A. Rouleaux, horse; *B.* Autoagglutination, dog; *C.* Rubricytosis (metarubricyte and rubricyte) and polychromatophilic erythrocyte, dog; *D.* Ghost erythrocytes, dog; *E.* Hypochromic erythrocytes of Fe deficiency, dog; *F.* Reticulocytes, NMB vital stain, dog; *G.* Reticulocytes (coarse and fine punctate), NMB vital stain, cat; *H. Anaplasma marginale,* cow; *I. Anaplasma centrale,* cow; *J. Babesia canis,* dog; *K. Babesia gibsoni,* dog (from ASVCP slide contributed by Dr. A.R. Irizarry-Rovira et al., 1999); *L. Cytauxzoon felis,* cat; *M. Eperythrozoon wenyonii,* cow (from ASVCP slide contributed by Dr. E.G. Welles et al., 1993); *N. Haemobartonella canis,* dog; *O. Haemobartonella felis,* cat; *P. Theileria buffeli,* cow; *Q.* Basophilic stippling of plumbism, dog; *R.* Distemper inclusions, dog (from ASVCP slide contributed by Dr. D.C. Bernreuter, 1981); *S.* Distemper inclusions, Diff-Quik stain, dog (from ASVCP slide contributed by Dr. J.R. Duncan, 1981); *T.* Heinz bodies, cat; *U.* Heinz bodies, NMB vital stain, cat; *V.* Hemoglobin crystal, dog; *W.* Howell-Jolly bodies, dog; *X.* Ringed Howell-Jolly body, horse (bar = 5 μm).

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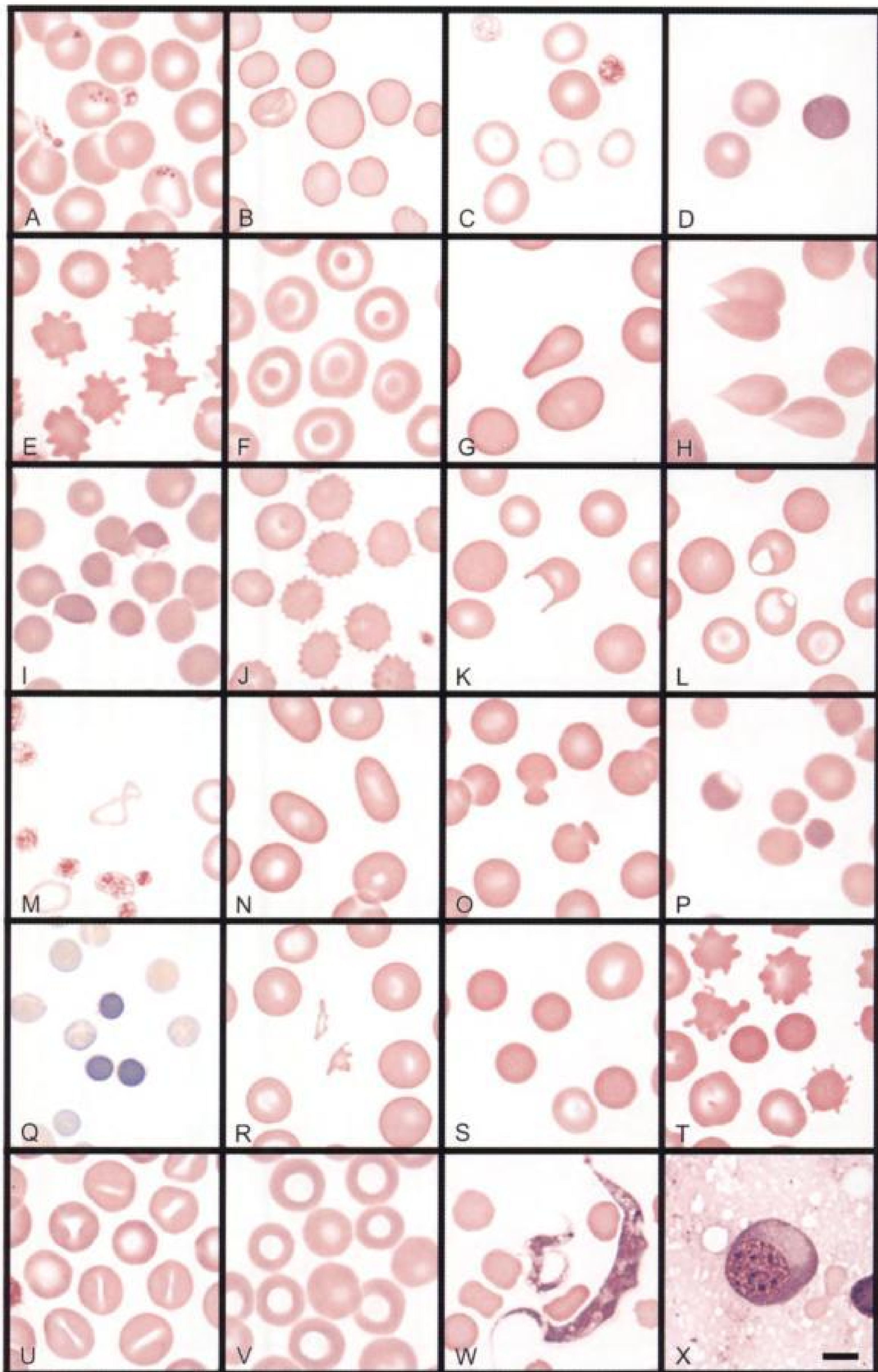

Plate 3. Photomicrographs of erythrocyte abnormalities. (All are from Wright-stained blood films unless otherwise stated) *A.* Siderocytes with siderotic granules, dog; *B.* Macrocyte and resulting anisocytosis, horse; *C.* Leptocytic, hypochromic microcytes of Fe deficiency, dog; *D.* Normochromic and polychromatophilic microcytes, portosystemic shunt, dog; *E.* Acanthocytes, dog; *F.* Codocytes, dog; *G.* Dacrocyte, dog; *H.* Artifactual dacrocytes, dog; *I.* Eccentrocytes, horse; *J.* Echinocytes, dog; *K.* Keratocyte, dog; *L.* Prekeratocytes, dog; *M.* Folded, hypochromic, microcytic leptocyte of Fe deficiency, dog; *N.* Ovalocytes, dog; *O.* Pincered cells, dog; *P.* Pyknocyte (four o'clock position), horse; *Q.* Pyknocytes, NMB vital stain, horse; *R.* Schizocytes, dog; *S.* Spherocytes, dog; *T.* Acanthocytes and spherocytes, dog; *U.* Stomatocytes, dog (from ASVCP slide contributed by Dr. D.E. Brown et al., 1992); *V.* Torocytes, dog; *W. Trypanosoma theileri*, cow (from ASVCP slide contributed by Dr. H. Bender et al., 1989); *X.* Megaloblastic rubricyte, bone marrow, cat (bar = 5 µm).

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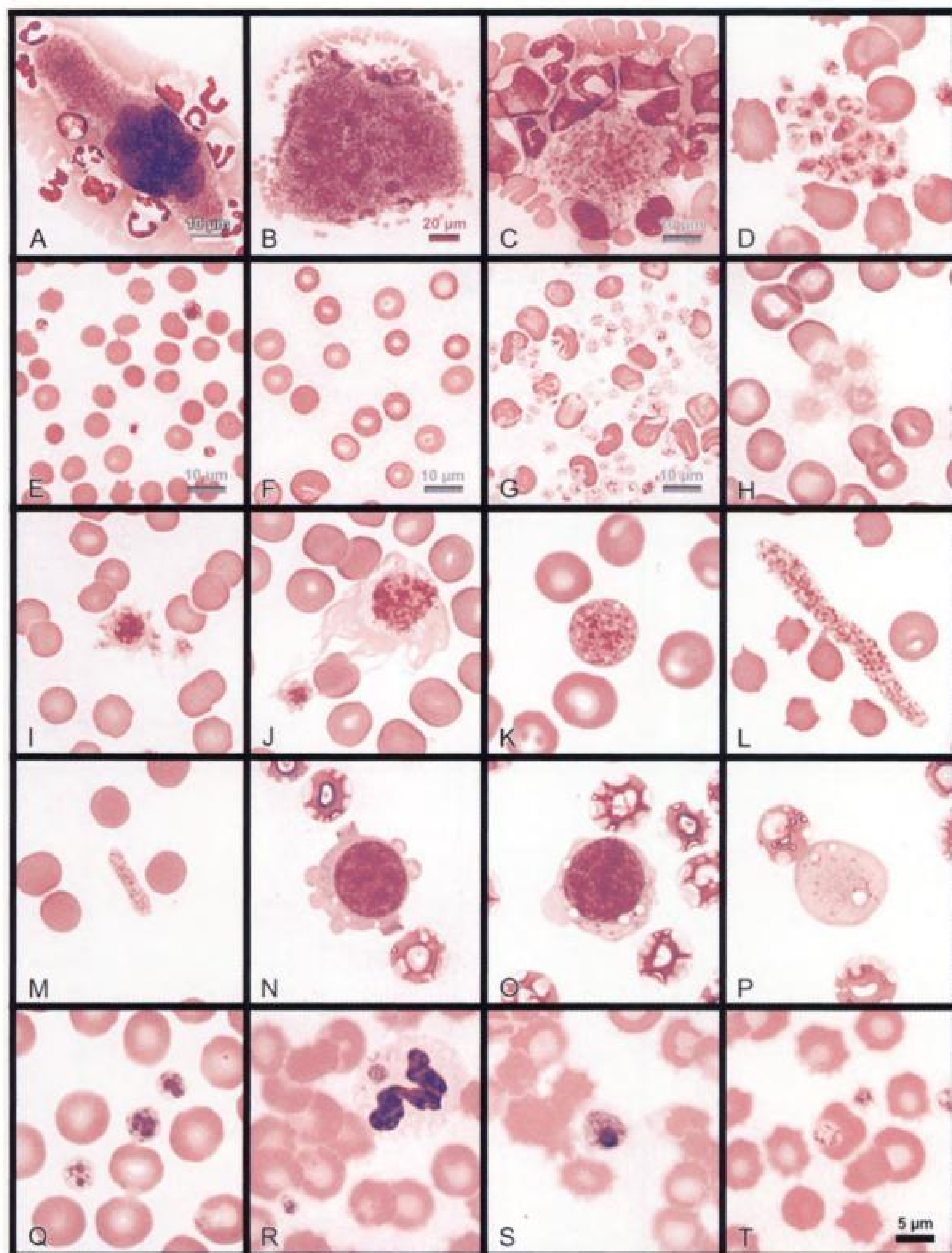

Plate 4. Photomicrographs of platelet abnormalities in Wright-stained blood films. (Scale bar in frame *T* applies to all other frames without a bar.)

A. Megakaryocyte in feathered edge of blood film, dog. *B.* Large platelet clump in feathered edge of blood film, cow. *C.* Smaller platelet clump surrounded by leukocytes and erythrocytes in feathered edge of blood film, cow. *D.* Small platelet clump in body of blood film, dog. *E.* Platelet density seen with a normal platelet concentration, dog. *F.* Platelet density seen with thrombocytopenia, dog. *G.* Platelet density seen with marked thrombocytosis due to essential thrombocythemia, dog (from ASVCP slide contributed by Dr. C.P. Mandell, 1987). *H.* Small cluster of four activated, degranulated platelets, cat. *I.* Activated giant platelet with pseudopods and centralized granules, cat. *J.* Giant activated platelet with pseudopods and centralized granules, Cavalier King Charles spaniel. *K.* Giant platelet, unactivated, dog. *L.* Elongated platelet (proplatelet), cow. *M.* Elongated platelet (probable proplatelet), foal. *N.* Megakaryoblast with cytoplasmic blebs, megakaryocytic leukemia (M7), dog (from ASVCP slide contributed by Dr. J.B. Messick, 1989). *O.* Megakaryoblast with cytoplasmic granules and vacuoles, megakaryocytic leukemia (M7), dog (from ASVCP slide contributed by Dr. J.B. Messick, 1989). *P.* Abnormal giant and hypogranular platelet associated with megakaryocytic leukemia (M7), dog (from ASVCP slide contributed by Dr. J.B. Messick, 1989). *Q. Ehrlichia platys* morulae in three platelets, dog (from ASVCP slide contributed by Dr. S. Gaunt, 1986). *R.* Platelet near a neutrophil containing a morula of *Ehrlichia ewingii*, showing similarity of size but different staining, dog. *S.* Platelet containing a probable fragment of nuclear material that can be mistaken for an organism, dog. *T.* Platelet overlying an erythrocyte, sometimes mistaken for an erythrocyte inclusion, dog.

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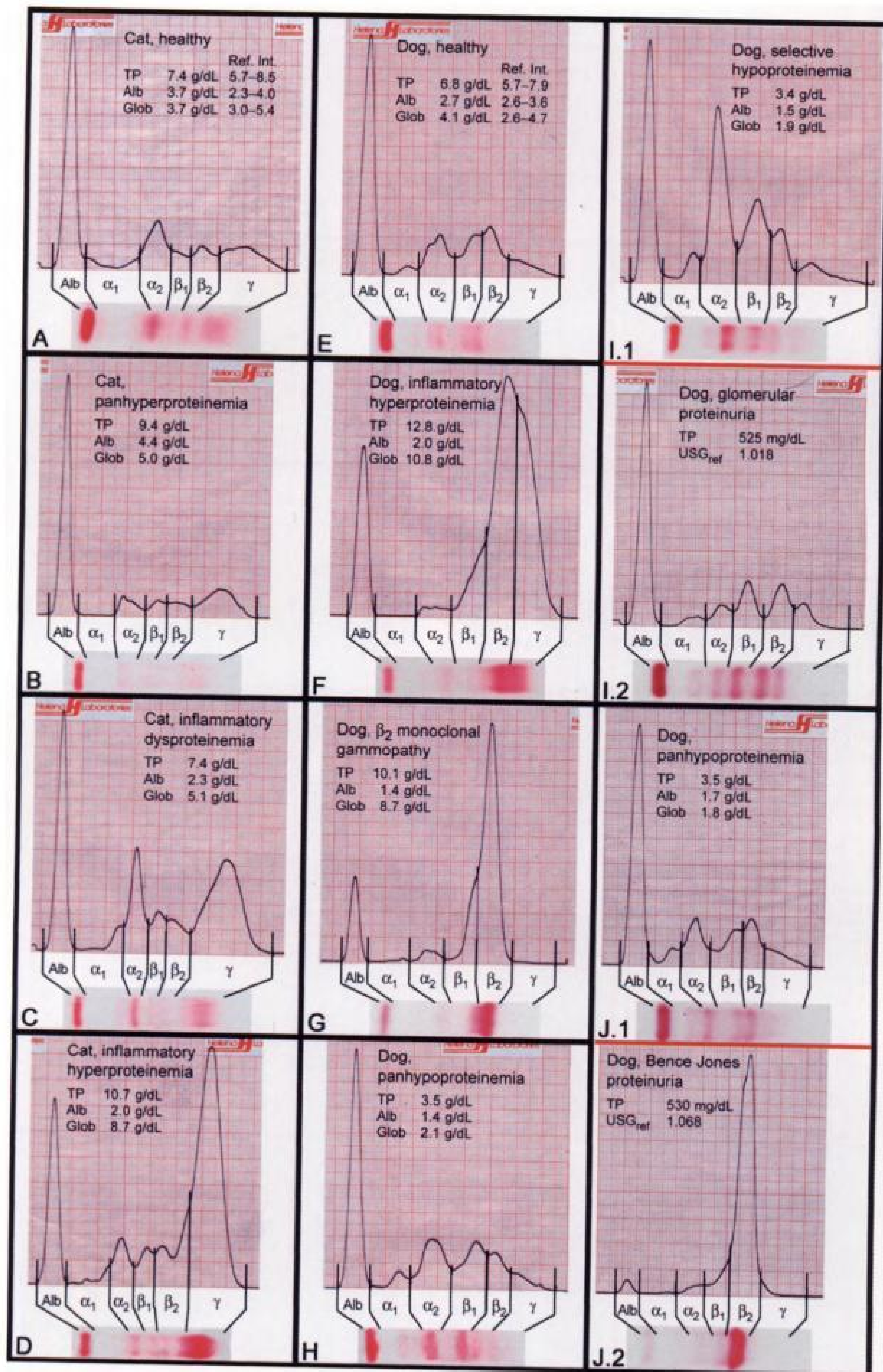

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Plate 5. SPE densitometer tracings, cellulose acetate strips, and serum protein concentrations from dogs and cats. Reference intervals for total protein, albumin, and globulin concentrations for cats and dogs are in A and E, respectively.

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.

B. *Cat, panhyperproteinemia.* The densitometer tracing is within expected results for a healthy cat but is found in a hyperproteinemic sample. Thus, protein concentrations are increased proportionately (panhyperproteinemia) and are consistent with hemoconcentration due to dehydration.

C. *Cat, inflammatory dysproteinemia.* The densitometer tracing shows a selective pattern—relatively less albumin compared to the globulin regions. Even though total globulin concentration is WRI, there are relatively more of α_2-globulin and γ-globulin fractions compared to the other globulin fractions. The increased α_2-globulin region is probably due to increased concentrations of haptoglobin or α_2-macroglobulin (positive acute phase proteins). The increased γ-globulin region is broad-based and is thus due to a polyclonal gammopathy (probably mostly IgG). Overall, the dysproteinemia is a delayed-response pattern caused by an inflammatory process of more than 7 days duration.

D. *Cat, inflammatory hyperproteinemia.* The densitometer tracing shows a selective pattern—relatively less albumin compared to the globulin regions. The hyperglobulinemia is due to increased γ-globulin concentration. The increased γ-globulin region is narrow and thus could be a monoclonal gammopathy or a polyclonal gammopathy with restricted migration. In this case, a post mortem diagnosis of feline infectious peritonitis and the absence of B-lymphocyte neoplasia indicated that the hyperproteinemia, hypoalbuminemia, and hyperglobulinemia were due to chronic inflammation.

E. *Dog, 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.

F. *Dog, inflammatory hyperproteinemia.* The densitometer tracing shows a selective pattern—relatively less albumin compared to the globulin regions. The hyperglobulinemia is due to increased β_2- and γ-globulin concentrations. This broad-based region represents a pronounced polyclonal gammopathy. In this case, clinical signs and an extremely high titer to *Ehrlichia canis* indicated that the hyperproteinemia was due to a chronic rickettsial (bacterial) infection.

G. *Dog, β_2 monoclonal gammopathy.* The densitometer tracing shows a selective pattern—relatively less albumin compared to the globulin regions. The hyperglobulinemia is due to increased β_2-globulin concentration. The β_2-globulin region is contains a narrow peak and an anodal shoulder. The combination of narrow β_2-globulin region and an apparent decrease in the γ-globulin concentration is indicative of a monoclonal gammopathy of a non-IgG immunoglobulin. This dog's hyperproteinemia was due to a myeloma and the serum IgA concentration was markedly increased.

H. *Dog, panhypoproteinemia.* The densitometer tracing is within expected results for a healthy dog but is found in a hypoproteinemic sample. Thus, protein concentrations are decreased proportionately (panhypoproteinemia). Causes of panhypoproteinemia include acute blood loss, maldigestive and malabsorptive disorders, starvation, cachexia, and occasionally hepatic failure. This dog had intestinal lymphoma.

I. *Dog, selective hypoproteinemia and glomerular proteinuria*

1. *Serum.* The densitometer tracing shows a selective pattern—relatively less albumin compared to the globulin regions. In a hypoproteinemic sample, this selective pattern indicates that albumin concentration is decreased more than some globulin concentrations. Even though the total globulin concentration is decreased, the relative excess of α_2-globulin region indicates that concentrations of other globulin fractions decreased more than the α_2-globulin concentration. This pattern is indicative of protein-losing nephropathy in which the glomerular filtration barrier has become more permeable to plasma proteins because of glomerulonephritis or glomerular amyloidosis. In such cases, there is a relative excess of the α_2-globulin region because α_2-macroglobulin is too large to pass through the filtration barrier but smaller proteins can. Note that even though there is hypoproteinemia, hypoalbuminemia, and hypoglobulinemia, there was not truly a panhypoproteinemia because the α_2-globulin concentration was not decreased.
2. *Urine.* The densitometer tracing shows that most urine proteins are in the albumin region, consistent with a protein-losing nephropathy with a glomerular proteinuria. Note that the proteinuria is a selective proteinuria because the urine protein pattern is not the same as the dog's serum protein pattern.

J. *Dog, nonselective hypoproteinemia and Bence Jones proteinuria*

1. *Serum.* The densitometer tracing is within expected results for a healthy dog but is found in a hypoproteinemic sample. Thus, protein concentrations are decreased proportionately (panhypoproteinemia). Causes of panhypoproteinemia include acute blood loss, maldigestive and malabsorptive disorders, starvation, cachexia, and occasionally hepatic failure. This dog had multicentric lymphoma with Mott cells and its hypoproteinemia was probably due to multiple processes.
2. *Urine.* Most urine proteins are in the β_2-globulin region, consistent with migration of immunoglobulin light chains. The Bence Jones urine test was positive; i.e., urine supernatant was initially clear, formed precipitate at 40°–60°C, cleared at 100°C, and then appearances reversed as the sample returned to room temperature. Note that the proteinuria is a selective proteinuria since the urine protein pattern is not the same as the dog's serum protein pattern and represents one type of a prerenal proteinuria (see Chap. 8).

Note: The serum total protein and albumin concentrations were measured by biuret and BCG methods, respectively. The serum globulin concentrations were calculated by subtraction from the measured values. When the electrophoresis strips were scanned, the densitometer was set so that the darkest protein band in the sample caused the maximum deflection of the tracing pen. Hyperproteinemic samples were diluted (either 1:2 or 1:4) prior to electrophoresis so that there was a more linear relationship between quantity of protein in the darkest band and the amount of light that passes through the strip. The urine total protein concentrations were measured by the Coomassie brilliant blue assay. The urine samples were concentrated 10-fold prior to electrophoresis.

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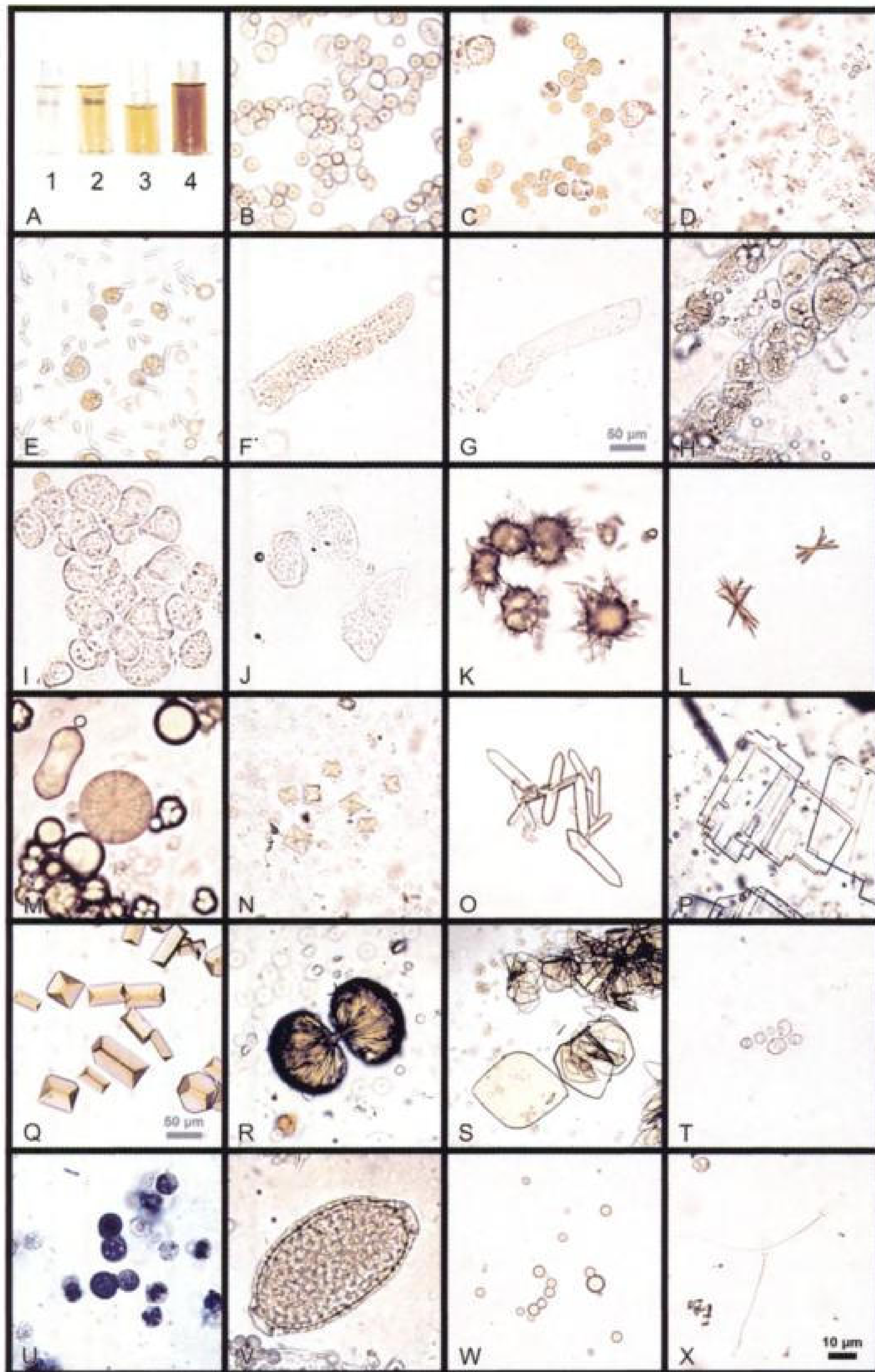

Plate 6. Photograph of urine *(A)* and photomicrographs of urine sediment findings *(B-X)*. Sediment was unstained except where noted *(U)*. All photomicrographs were taken using a high-dry objective (use approximate scale bar in frame *X*) except for *G* and *Q*, which were taken using a 10× objective (use gray scale bars).

A. USG_{ref} and osmolality of urine samples with different gross appearances and colors demonstrating that appearance does not necessarily predict USG_{ref} or solute concentration: (1) colorless, USG_{ref} = 1.014, osmolality = 410 mosmol/kg H_2O; (2) light yellow, USG_{ref} = 1.014, osmolality = 531 mosmol/kg H_2O; (3) yellow, USG_{ref} = 1.013, osmolality = 292 mosmol/kg H_2O; (4) dark yellow, USG_{ref} = 1.023, osmolality = 551 mosmol/kg H_2O. *B.* Leukocytes and erythrocytes. *C.* Erythrocytes and few lewkocytes. *D.* Small bacterial cocci, sometimes in short chains. *E.* Large bacterial rods and several leukocytes (courtesy of Dr. Don Schmidt, University of Missouri). *F.* Granular cast. *G.* Hyaline cast (low magnification). *H.* Epithelial cell cast. *I.* Epithelial cell cluster (probably transitional epithelial cells). *J.* Squamous epithelial cells. *K.* Ammonium biurate crystals. *L.* Bilirubin crystals. *M.* Calcium carbonate crystals. *N.* Calcium oxalate dihydrate crystals and bacteria. *O.* Calcium oxalate monohydrate crystals. *P.* Cholesterol crystals (courtesy of Dr. Don Schmidt). *Q.* Struvite crystals (low magnification). *R.* Sulfa crystals and erythrocytes (courtesy of Dr. Don Schmidt). *S.* Uric acid crystals. *T.* Yeast. U. *Blastomyces* sp. and several neutrophils, new methylene blue stain. *V.* *Capillaria* sp. ovum (courtesy of Dr. Don Schmidt). *W.* Lipid droplets. *X.* Sperm.

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Chapter 4

ERYTHROCYTES

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Table 4.1. Abbreviations and symbols in Chapter 4

»	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
2,3-DPG	2,3-diphosphoglycerate
AID	anemia of inflammatory disease
AIHA	autoimmune hemolytic anemia
ALA	aminolevulinic acid
ASVCP	American Society for Veterinary Clinical Pathology
ATP	adenosine triphosphate
Bc	conjugated bilirubin
BFU-E	burst forming unit–erythroid
BLV	bovine leukemia virus
Bu	unconjugated bilirubin
C3	complement protein 3
C5b-9	complement membrane attack complex
Cb_5R	cytochrome-b_5 reductase
CFU-E	colony forming unit–erythroid
CRP	corrected reticulocyte percentage
DEA	dog erythrocyte antigen
DNA	deoxyribonucleic acid
DPG	diphosphoglycerate
ECF	extracellular fluid
EIA	equine infectious anemia
EIAV	equine infectious anemia virus
Epo	erythropoietin
ESAIg	erythrocyte surface-associated immunoglobulin
EVH	extravascular hemolysis
FAD	flavin adenine dinucleotide
Fe	iron, either Fe^{2+} or Fe^{3+}
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FeLV	feline leukemia virus
fT_4	free thyroxine
G6PD	glucose-6-phosphate dehydrogenase
GI	gastrointestinal
GR	glutathione reductase
GSH	glutathione
Hct	hematocrit
Hgb	hemoglobin (iron in Fe^{2+} state)
Hgb-Fe^{3+}	methemoglobin
HMP	hexose monophosphate
IHA	immune hemolytic anemia
IIHA	idiopathic immune hemolytic anemia
IL-x	interleukins (x for Arabic numbers)
INF	interferon
IVH	intravascular hemolysis
M6	erythroleukemia (form of acute myeloid leukemia)
M6-Er	erythroleukemia with erythroid predominance

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MCH	mean cell hemoglobin
MCHC	mean cell hemoglobin concentration
MCV	mean cell volume
MDS	myelodysplastic syndrome
MDS-Er	myelodysplastic syndrome with erythroid predominance
MPD	myeloproliferative disease
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NI	neonatal isoerythrolysis
NMB	new methylene blue
nRBC	nucleated erythrocyte
O_2	oxygen
P_aCO_2	partial pressure of carbon dioxide in arterial blood
P_aO_2	partial pressure of oxygen in arterial blood
PCR	polymerase chain reaction
PFK	phosphofructokinase
PK	pyruvate kinase
RBC	red blood cell, erythrocyte
RC	reticulocyte concentration
SI	Système International d'Unités
TGF-β	tumor growth factor-beta
TIBC	total iron-binding capacity
TNF	tumor necrosis factor
TP	total protein
TSH	thyroid stimulating hormone, thyrotropin
tT_3	total triiodothyronine
tT_4	total thyroxine
UIBC	unbound iron-binding capacity
WRI	within reference interval

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

PHYSIOLOGIC PROCESSES

I. Erythron: all erythroid cells in an animal; includes precursors and erythrocytes in blood vessels and sinuses of spleen, liver, and marrow (Fig. 4.1)
   A. Erythrocyte precursors
      1. Erythropoiesis is part of hematopoiesis, which is a complex system involving stem cells and cytokines (see Fig. 3.1). Committed stem cells (e.g., CFU-E) respond to Epo by either dividing or by differentiating toward rubriblasts.
      2. Renal cells produce Epo in response to renal hypoxia. Renal hypoxia may be due to anemia, poor oxygenation of blood (e.g., high altitude, pulmonary disease), or poor renal perfusion.
      3. After a CFU-E differentiates into a rubriblast, two major cell processes occur.
         a. Cells undergo mitoses to produce more and smaller cells. As [Hgb] increases in the developing cell, Hgb inhibits DNA synthesis and thus fewer mitoses occur.
         b. Cells produce mRNA for synthesis of Hgb and cytoskeletal proteins.

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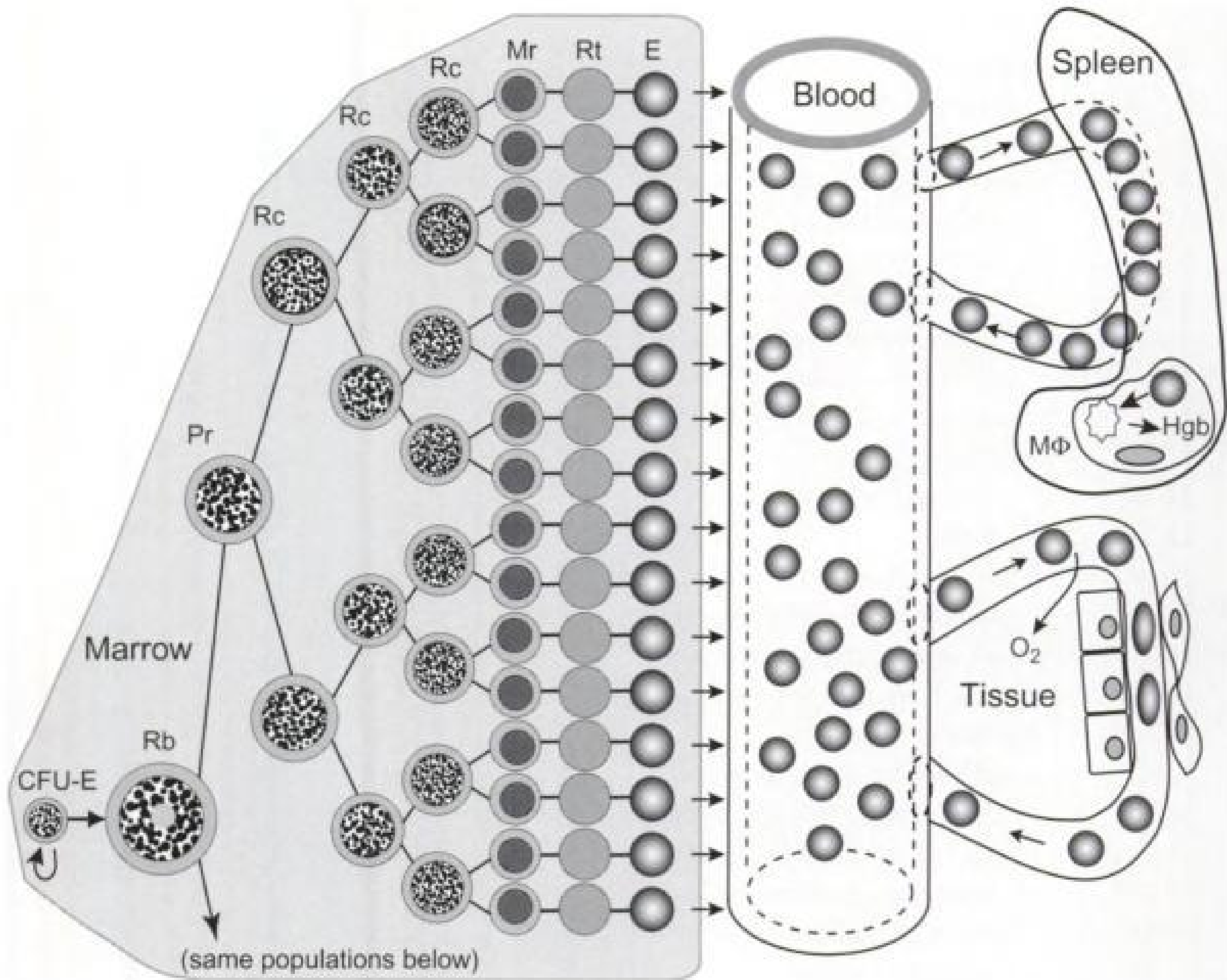

Fig. 4.1. Erythrocyte kinetics in health. (Pr = prorubricyte, Rc = rubricyte, Mr = metarubricyte, Rt = reticulocyte)

The erythron contains three major pools: erythrocyte precursors (mostly in marrow), blood erythrocytes, and splenic erythrocytes. After stimulation by Epo, CFU-E differentiate into rubriblasts (Rb) and the precursors proliferate (via mitosis) and mature until erythrocytes (E) are formed. An orderly maturation process results in a pyramidal population of erythroid cells (only the top half is shown). After release to blood, erythrocytes circulate in the vascular system to transport O_2 to tissues. A reserve pool of erythrocytes is sequestered in the spleen of most mammals. Senescent erythrocytes are destroyed by macrophages.

4. In the later stages of mammalian erythropoiesis, a metarubricyte nucleus is extruded and engulfed by a macrophage. The resulting anucleate cell is a reticulocyte.
5. When the nucleated erythrocyte loses its nucleus and ability to produce mRNA for protein synthesis, it soon loses the ability to produce Hgb and enzymes. The activities of erythrocytic enzymes are greatest in young erythrocytes and decrease slowly with age. Mitochondria deteriorate so that glycolysis becomes the ATP-producing pathway in most animals (except pigs that use an alternate energy pathway involving inosine).

B. Blood erythrocytes
   1. Major features of erythrocytes in different species are compared in Table 4.2.
   2. Erythrocyte destruction in health (senescence); about 100 million erythrocytes die/hour/pound body weight
      a. Old erythrocytes have very little metabolic machinery (enzymes) to keep themselves functional and deformable. Near death, they become more rigid, spheroid, and less able to pass through sinuses.

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Table 4.2. Comparison of blood erythrocytes in mature healthy animals

	Dogs	Cats	Horses	Cattle
Reticulocyte concentration (/μL)	50,000	40,000[a]	0	0
RBC concentration (× 10^6/μL)	6.8	7.5	8.0	7.0
RBC life span (days)	100	70	150	150
RBCs in blood[b] (× 10^{12})	5.4	2.1	400	280
RBC diameter (μm)	7.0	5.8	5.5	5.8
RBC volume (fL)	70	45	45	52
RBC central pallor	prominent	mild	none to mild	mild to moderate

Note: All numbers represent approximate averages to illustrate similarities and differences; they are not reference intervals.

[a] Counting only aggregate reticulocytes.

[b] Based on 40 mL blood/lb body weight and average-sized animals.

b. Age-related changes in erythrocyte membranes expose antigens that are bound by naturally occurring antibodies that may mediate erythrocyte destruction.[1]

C. Splenic erythrocytes

1. Spleens of dogs, horses, and cattle have sinusoids and red pulp that are full of erythrocytes; 50%–60% of a horse's erythrocytes can be within its spleen. Damaged or less deformable erythrocytes are removed by macrophages that are adjacent to sinusoids or present in the red pulp. When splenic contraction occurs, erythrocytes are forced into the systemic blood.
2. Cat spleens are thought to have closed circulation (blood does not flow through red pulp), which is less efficient at removing damaged erythrocytes. They also do not have large reserve erythrocyte pools.
3. Most metarubricytes and about 50% of reticulocytes that are released from marrow each day are temporarily trapped in mammalian spleens (except cats).

II. Erythrocyte kinetics

A. Blood erythrocyte concentrations are established by the relative rates of erythrocyte production, shifting of erythrocytes to and from splenic sinuses, and erythrocyte destruction.

B. Erythrocyte production depends on the degree and duration of Epo stimulus and the ability of precursor cells to respond to Epo.

III. Hgb: structure, function, synthesis, degradation

A. Hgb structure

1. Hgb is a tetramer with each globin linked to a separate heme that binds O_2. Globins are polypeptides, and in mature healthy mammals, each Hgb molecule contains two α chains and two β chains. Approximately 95% of an erythrocyte is Hgb on a dry weight basis.
   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, there should be increases in both heme and globin syntheses.
   b. If synthesis of a globin chain is decreased, an animal has a thalassemia.
   c. If there are gene mutations that produce abnormal amino acid sequences in the globins, an animal has a hemoglobinopathy.

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2. Fe in heme is in the ferrous state (Fe^{2+}) and is kept in the reduced state by enzymatic reactions catalyzed by Cb_5R (NADH-methemoglobin reductase, commonly called just 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 glutathione reductase and catalase.

B. Hgb function
1. Hgb transports O_2 from lungs to tissues. In health, Hgb is 100% saturated with O_2 in arterial blood. Hgb-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 erythrocytes, 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 additional conversion of CO_2 to HCO_3^-. When erythrocytes 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 erythrocytes, 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. Hgb synthesis occurs in erythrocyte precursors (rubriblasts through reticulocytes) in a series of reactions (Fig. 4.2).
2. ALA synthetase is the major rate-limiting enzyme. It requires vitamin B_6 as a cofactor, and it is inhibited by higher heme concentrations.

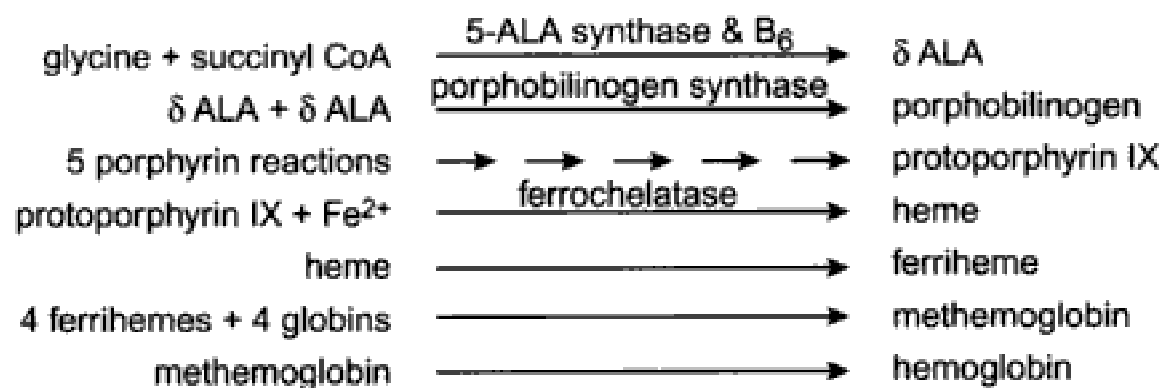

Fig. 4.2. Hemoglobin synthesis and degradation.
- *Hemoglobin synthesis in erythrocyte precursors:* The synthesis of Hgb has three major components: (1) series of porphyrin reactions, (2) incorporation of Fe^{2+} into protoporphyrin IX to form heme, (3) and binding of four ferriheme and four globin molecules to form hemoglobin.
- *Hemoglobin degradation in macrophages:* In health, senescent erythrocytes are engulfed by macrophages and heme is split from globin chains. Heme is degraded to bilirubin, Fe^{2+}, and CO. The globin chains are degraded to amino acids.

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3. 5-ALA synthetase, porphobilinogen synthase, ferrochelatase, and coproporphyrinogen oxidase are inhibited by lead.
   a. In lead toxicity, inhibition of these enzymes leads to higher concentrations of heme precursors in erythrocytes. Collectively, porphobilinogen through protoporphyrin IX are called porphyrins.
   b. Porphyria is a condition in which there are increased concentrations of porphyrins in erythrocytes, plasma, or urine and it can be an acquired (as in lead toxicity) or a congenital condition. Animals with porphyria are prone to photosensitivity.
4. Maturation rate of erythrocyte precursors is affected by [Hgb] in their cytoplasms. If Hgb synthesis is incomplete, additional mitoses during a cell's development will result in the production of smaller erythrocytes.

D. The type of Hgb synthesized in an individual can vary with the animal's age and health. Fetal Hgb (which contains two α and two γ chains) is replaced by adult Hgb during fetal development in dogs, cats, and horses but during the first postnatal month in ruminants.[1]

E. Hemoglobin degradation and bilirubin metabolism: After erythrocyte death in macrophages of spleen, liver, or marrow, Hgb is degraded to Bu, amino acids, and Fe (Figs. 4.2 and 4.3). Bu and Bc are excreted or degraded, whereas the amino acids and Fe are recycled.

IV. Fe
   A. Total body Fe is distributed in three major sites in health: (1) about 50%–70% in erythrocyte Hgb, (2) about 25%–40% in storage, and (3) the remainder in other molecules (e.g., myoglobin, cytochromes, enzymes).[2]
   B. Physiologic processes or concepts (Fig. 4.4)

V. Mature erythrocyte metabolism (Fig. 4.5)
   A. Glucose is the major energy source for mature erythrocytes in most species.
   B. Pig erythrocytes lack a functional glucose transporter and use inosine instead of glucose.

MORPHOLOGIC FEATURES OF ERYTHROCYTES: CLINICAL SIGNIFICANCE AND PATHOGENESES

I. General features
   A. Discocytes: Mature erythrocytes of each domestic mammal species are disks with different degrees of biconcavity that creates central pallor (Table 4.2).
   B. Rouleau, rouleaux (pl.): aggregate of erythrocytes resembling a "stack of coins" (see Plate 2.*A*)
      1. Common in some species (especially horses)
      2. Formation is affected by erythrocyte factors (shape and membrane composition), albumin factors (glycation), globulin factors (charge, size, and number), and pH (affects cell and protein charges).
      3. Rouleaux tend to occur if there is hyperglobulinemia or hyperfibrinogenemia.
   C. Agglutination: aggregation or clumping of erythrocytes into grape-like clusters (see Plate 2.*B*)
      1. Autoagglutination is seen in some immune hemolytic anemias, in cold agglutinin disorders, and occasionally in animals without evidence of hemolysis. The agglutinin

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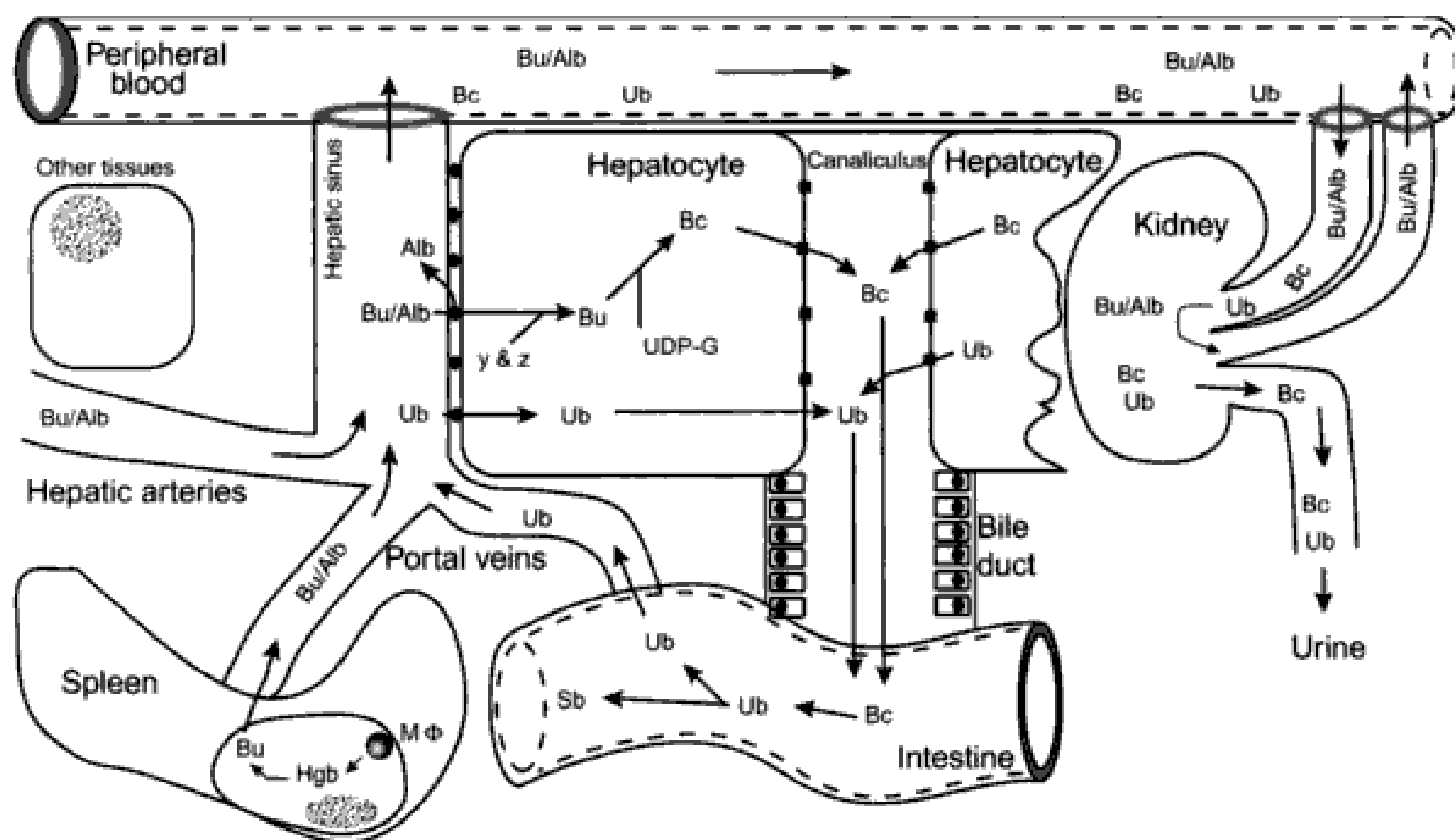

Fig. 4.3. Bilirubin metabolism. (Bu/Alb = Bu associated with albumin, MΦ = macrophage, UDP-G = uridine diphosphoglucoronide, Sb = stercobilinogen, Ub = urobilinogen)

- In health, erythrocyte destruction in macrophages of spleen, liver, or marrow results in Bu formation. Small and usually clinically insignificant amounts of Bu are formed from heme degradation associated with ineffective erythropoiesis and degradation of other heme-containing molecules (catalase, peroxidase, cytochromes). As Bu leaves a macrophage, it forms a noncovalent association with albumin and is transported to hepatocytes. Bu is relatively H_2O insoluble prior to binding to albumin (Alb).
- When Bu enters the liver and its protein-permeable sinuses, it probably binds to hepatocyte membrane receptors, enters hepatocytes without albumin, and binds to y-protein (ligandin) or z-protein (fatty-acid binding protein). Bu probably enters hepatocytes by a passive but facilitated process; binding proteins enhance the process by reducing the efflux of Bu back to the sinusoidal plasma.
- Within hepatocytes, Bu is conjugated with glucuronide (glucose in horses) to form bilirubin monoglucoronide or bilirubin diglucuronide, which collectively are called Bc.
- 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 bile acids.
- Bc in bile enters the intestine and is degraded to urobilinogen (colorless). Urobilinogen can be passively absorbed in the intestine and then enter hepatocytes for excretion in bile, or it can bypass the liver and be excreted in urine. Urobilinogen can also be degraded to stercobilinogen (dark brown) and excreted in feces.
- If Bc escapes hepatocytes and enters blood, it can pass through a glomerular filtration barrier and be excreted in urine. Because albumin does not pass through the glomerular filtration barrier of most mammals, Bu/Alb does not enter urine in those animals.

(a substance causing the agglutination) is typically a cold antibody, i.e., an antibody that has maximal activity at 4°–20°C. The erythrocyte clusters formed by autoagglutination must be differentiated from rouleaux, which classically appear as stacks of erythrocytes but can appear as piles or fallen stacks of coins. Rouleau should disperse into individual erythrocytes when blood protein concentration is lowered by diluting blood with saline (1:1 dilution), whereas autoagglutination does not disperse. Occasionally, not all erythrocytes disperse after dilution when rouleaux are numerous.

2. Heparin may induce agglutination of equine erythrocytes.

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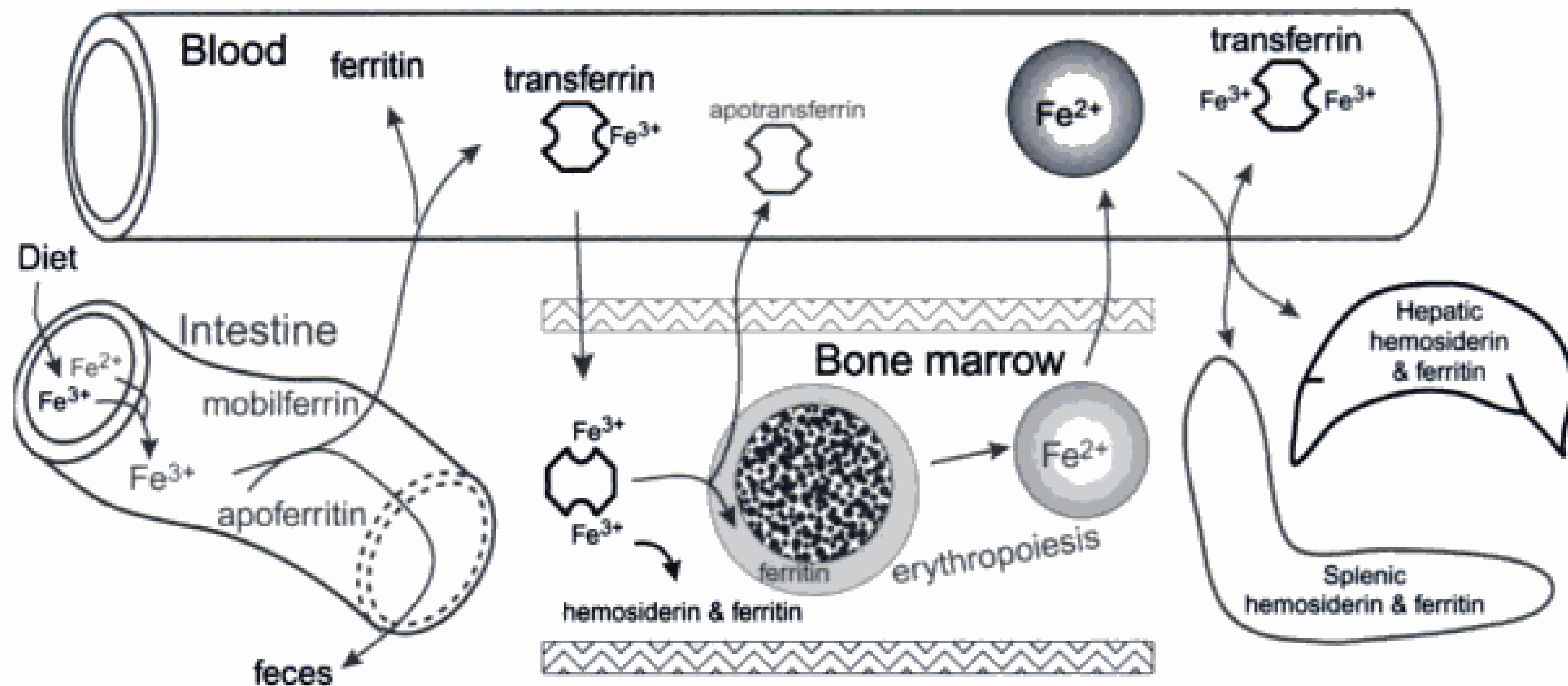

Fig. 4.4. Fe kinetics in healthy animals.

- *Absorption:* Diets of domestic mammals may contain Fe^{2+} or Fe^{3+}. The process of intestinal absorption is not completely understood but involves gastric acid, intestinal mucin, binding to membrane integrins, mobilferrin (a shuttle protein), and apoferritin. Apoferritin in mucosal epithelial cells binds to Fe^{3+} to form mucosal ferritin, which appears to be lost into the intestine when mucosal cells are sloughed. 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.
- *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 33% of transferrin's Fe-binding sites are occupied by Fe. Many cells have transferrin receptors, especially marrow erythroid cells and hepatocytes.
- *Use in erythroid cells:* After transferrin binds to and enters erythroid precursors, Fe^{3+} dissociates from apotransferrin and binds to cytoplasmic apoferritin (to form ferritin) or is incorporated into heme (Fe^{2+}) and then hemoglobin. Most apotransferrin escapes degradation and is returned to plasma. In health, about 50%–70% of total body Fe is within erythrocytes.
- *Storage:* Fe^{3+} is stored in two protein-Fe complexes: ferritin (plasma and tissue) and hemosiderin. In health, about 25%–40% of total body Fe is within storage forms. Young animals (especially neonates) have low amounts of stored Fe.
  - Ferritin consists of apoferritin complexed with Fe^{3+} and is a relatively soluble, mobile source of Fe^{3+}; there are several forms of apoferritin due to various combinations of H or L subunits. Plasma ferritin is a glycosylated polymer that is relatively Fe-poor. Tissue ferritin, which is not glycosylated and is relatively Fe-rich, is produced by many cells, primarily macrophages, hepatocytes, intestinal mucosal epithelial cells, and erythroid precursors. Synthesis of tissue ferritin by hepatocytes and macrophages is increased by inflammation (ferritin is a positive acute phase protein) and when there is increased Fe storage.
  - Hemosiderin is a relatively insoluble, poorly mobile source of Fe^{3+} and represents the major storage form of Fe. Hemosiderin is a globular protein that is found primarily in macrophages of spleen, liver, and marrow of most mammals. A healthy cat's marrow does not have enough hemosiderin to be detected by routine staining methods.
- *Tissue forms:* A relatively small quantity of Fe is present in myoglobin, catalase, peroxidases, and cytochromes.

3. Agglutination may interfere with electronic or optical evaluation of erythrocytes when a group of erythrocytes passes through the counting chamber as "one large cell." In such cases, the measured MCV and erythrocyte concentrations are erroneous (unless programs exclude outlier values), as are the values calculated from those measured values.

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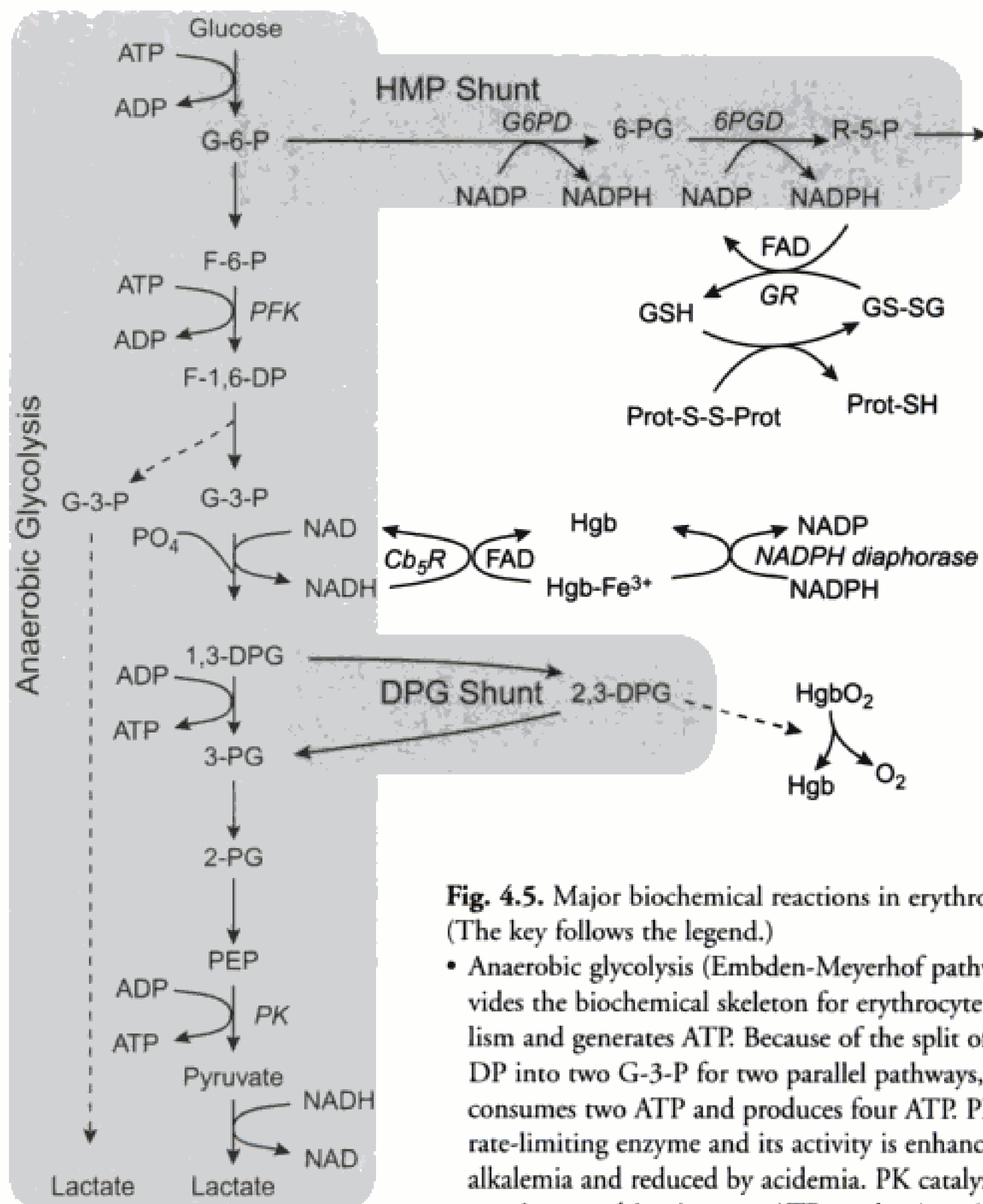

Fig. 4.5. Major biochemical reactions in erythrocytes. (The key follows the legend.)

- Anaerobic glycolysis (Embden-Meyerhof pathway) provides the biochemical skeleton for erythrocyte metabolism and generates ATP. Because of the split of F-1,6-DP into two G-3-P for two parallel pathways, glycolysis consumes two ATP and produces four 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 a net ATP production via anaerobic glycolysis.
- The HMP shunt (pentose shunt, 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 erythrocytes and are used to maintain Hgb and other proteins in a functional reduced state. G6PD is the first enzyme of the shunt.
- Cb_5R (methemoglobin reductase) uses FAD and NADH to catalyze the conversion of Hgb-Fe^{3+} to Hgb.
- NADPH diaphorase 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.
- DPG shunt (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.

(1,3-DPG = 1,3-diphosphoglycerate, 2-PG = 2-phosphoglycerate, 2,3-DPG = 2,3-diphosphoglycerate, 3-PG = 3-phosphoglycerate, 6-PG = 6-phosphogluconate, 6PGD = 6-phosphogluconate dehydrogenase, ADP = adenosine diphosphate, ATP = adenosine triphosphate, Cb_5R = cytochrome-b_5 reductase, DPG = diphosphoglycerate, F-1,6-DP = fructose-1,6-diphosphate, F-6-P = fructose-6-phosphate, G-3-P = glyceraldehyde-3-phosphate, G-6-P = glucose-6-phosphate, G6PD = glucose-6-phosphate dehydrogenase, GSH = glutathione [reduced], GS-SG = glutathione disulfide, Hgb = deoxyhemoglobin, Hgb-Fe^{3+} = methemoglobin, Hgb-O_2 = oxyhemoglobin, HMP = hexose monophosphate, MR = methemoglobin reductase, NAD = nicotinamide adenine dinucleotide, NADH = reduced nicotinamide adenine dinucleotide, NADP = nicotinamide adenine dinucleotide phosphate, NADPH = reduced nicotinamide adenine dinucleotide phosphate, PEP = phosphoenolpyruvate, PFK = phosphofructokinase, PK = pyruvate kinase, PO_4 = phosphate, Prot-SH = protein with reduced sulfhydryl groups, Prot-S-S-Prot = protein with disulfide bridges, R-5-P = ribulose-5-phosphate)

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D. Rubricytosis (normoblastemia) (see Plate 2.*C*)
   1. Definition: increased concentration of nRBCs in blood. Usually, most are metarubricytes, a few may be rubricytes, and younger precursors are rarely seen.
   2. Rubricytosis is common in regenerative anemias (i.e., appropriate rubricytosis) but also may be seen in nonregenerative anemias and in nonanemic animals (inappropriate rubricytosis). Therefore, rubricytosis should not be considered a consistently reliable indicator of a responsive marrow.
      a. Appropriate rubricytosis: rubricytosis concurrent with regenerative anemia (with reticulocytosis)
         (1) Occurs during accelerated erythropoiesis; not only is there increased release of reticulocytes, but also an increased release of nRBCs
         (2) Seen in regenerative anemias of dogs, cats, cattle, and pigs; occasionally seen in horses
      b. Inappropriate rubricytosis: rubricytosis concurrent with nonregenerative anemia or in the absence of anemia
         (1) Occurs primarily when there is a loss of the finely controlled release of nRBCs from marrow or other erythropoiesis sites; nRBCs escape from marrow or other erythropoiesis sites before maturing to reticulocytes or without nuclear extrusion.
         (2) Disorders or conditions that cause inappropriate rubricytosis
            (a) Marrow damaged by necrosis, inflammation, neoplasia, or hypoxia: Nucleated erythrocytes gain entrance into marrow sinuses through damaged sinusoidal endothelium.
            (b) Extramedullary hematopoiesis (especially splenic): may allow release of cells before nuclear extrusion
            (c) Splenic contraction: Splenic blood contains nucleated erythrocytes that are completing maturation.
            (d) Splenectomy: The few nRBCs that are normally released from marrow are not "caught" by the spleen.
            (e) Lead poisoning in dogs, perhaps the result of damage to marrow sinuses

II. Erythrocyte color
   A. Central pallor: pale region near the center of erythrocyte that is due to the relative thinness of the area which is created by the cell's biconcave shape
      1. Increased central pallor is usually indicative of hypochromasia.
      2. Decreased central pallor usually indicates abnormally shaped erythrocytes (poikilocytes, including spherocytes). It may also be seen near a blood film's feathered edge because of artifactual distortion of erythrocyte shape.
   B. Ghost cell: extremely pale-staining erythrocyte consisting primarily of cell membrane with only a small amount of residual cytoplasmic Hgb (see Plate 2.*D*)
      1. Ghost cells usually form as a result of complement-mediated intravascular hemolysis. Membrane attack complexes form membrane pores through which Hgb leaks out.
      2. Ghost cells may form *in vitro* as a result of smearing trauma. These artifactual ghost cells are often distorted.
   C. Hypochromic erythrocyte: poikilocyte with increased central pallor and more faintly stained Hgb than usual (see Plate 2.*E*)

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1. Hypochromasia is an increased number of hypochromic erythrocytes, which is usually reflected by a decreased MCHC.
2. Hypochromic erythrocytes result from a decreased intracellular [Hgb]. When visually evident, they usually are associated with Fe deficiency. However, hypochromasia based on MCHC alone is usually associated with the incomplete Hgb synthesis of immature erythrocytes (i.e., regenerative anemias).
3. Hypochromic erythrocytes of Fe deficiency are prone to structural changes such as irregular membranes, loss of circular shape, and fragmentation.

D. Polychromatophilic erythrocyte (polychromatophil): a non-nucleated, immature erythroid cell with cytoplasmic RNA (see Plate 2.*C*)
1. Polychromatophilic erythrocytes are the same cells as aggregate reticulocytes but visualized with a Wright stain. The cell's polychromasia (many colors) is the result of its cytoplasmic RNA (basophilic staining) and varying cellular Hgb concentrations (eosinophilic staining).
2. If there are increased numbers of polychromatophilic erythrocytes in a blood film, there is increased polychromasia that reflects accelerated erythropoiesis.

E. Reticulocyte: a non-nucleated, immature erythroid cell with cytoplasmic RNA (see Plate 2.*F* and *G*)
1. A reticulocyte's cytoplasmic RNA is visualized after staining with NMB stain or other vital stains. In cats, reticulocytes are grouped into two types (aggregate and punctate) based on the staining pattern of the RNA (see Chap. 2).
2. Reticulocytosis (increased blood reticulocyte concentration), like increased polychromasia, is an important indicator of accelerated erythropoiesis.

III. Erythrocyte parasites
A. Identifying features (Table 4.3)
B. Major aspects of the anemias caused by organisms are included in later sections.

IV. Inclusions other than parasites (Table 4.4)
A. Basophilic stippling (punctate basophilia) (see Plate 2.*Q*)
1. Basophilic stippling is the presence of fine to coarse, blue to dark purple dots of aggregated ribosomes dispersed within the erythrocyte cytoplasm. Basophilic stippling must be differentiated from siderotic granules.
2. Basophilic stippling is seen with regenerative anemias, especially in cattle.
3. When seen without corresponding polychromasia or reticulocytosis, or in nonanemic animals, plumbism is a common cause, especially in dogs. Lead inhibits pyrimidine 5'-nucleotidase that helps degrade nucleotides in RNA.

B. Distemper inclusions in dogs (see Plate 2.*R* and *S*)
1. Erythrocyte distemper inclusions are generally rare findings. They may appear as pink or pale blue amorphous inclusions of varying shapes and sizes.
2. Similar cytoplasmic inclusions are rarely found in blood neutrophils and lymphocytes.

C. Heinz body (see Plate 2.*T* and *U*)
1. Heinz bodies are aggregates of denatured Hgb caused by oxidative damage.
2. Heinz bodies are visualized with NMB stain as pale blue round structures associated with and sometimes protruding from erythrocyte membranes. In Wright-stained films, Heinz bodies have nearly the same staining features as normal Hgb but appear as slightly pale structures that create membrane defects or protrude from erythrocytes. Heinz bodies may detach from erythrocytes and occur as free bodies in a blood film.

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Table 4.3. Erythrocyte organisms: Identifying features and associated pathogenic processes

Organism	Identifying features[a]	Associated pathogenic processes
Anaplasma marginale	Marginal body is a small, dark-staining coccus about 0.5 μm diameter on the internal margin of erythrocytes; typically one organism per cell but may be multiple (Plate 2.*H*)	Immune hemolysis
Anaplasma centrale	Small dark-staining coccus about 0.5 μm diameter within erythrocytes; typically one organism per cell but may be multiple (Plate 2.*I*)	Immune hemolysis
Babesia spp.	Intracellular oval to teardrop or pear-shaped (pyriform) trophozoites (piroplasms); sizes vary with species (see text); typically pale blue with a darker outer membrane and a reddish purple eccentric nucleus (Plate 2.*J* and 2.*K*)	Several theories, including immune mechanisms, protease activity, decreased cell pliability, and oxidative damage
Cytauxzoon felis	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 (Plate 2.*L*)	Pathogenesis of the anemia may be multifaceted (anemia of inflammation, marrow damage, hemolysis)
Eperythrozoon spp.[b]	Rings, rods, or cocci on surface of erythrocytes; 0.3–1.0 μm diameter (Plate 2.*M*)	Immune hemolysis
Haemobartonella canis[b]	Typically thin chains of cocci on membrane that may form pleomorphic patterns (violin bow, figure eight, oval, cross); occasionally seen as individual cocci or rods (Plate 2.*N*)	Immune hemolysis
Haemobartonella felis[b]	Typically cocci (individual or in short chains); may form small rings or doughnuts (< 1 μm) on erythrocyte surface; stain blue-grey to pale purple (Plate 2.*O*)	Immune hemolysis
Theileria spp.	Highly pleomorphic piroplasms, including cocci, rings, rods, pears, and maltese crosses (Plate 2.*P*)	Several theories, including immune mechanisms, protease activity, decreased cell pliability, and oxidative damage

[a] Appearance as seen on a Wright-stained blood film unless stated otherwise.

[b] May be reclassified as members of the genus *Mycoplasma* based on gene sequences. (Neimark H. Johansson KE, Rikihisa Y, Tully JG. 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus Mycoplasma haemofelis*', '*Candidatus Mycoplasma haemomuris*', '*Candidatus Mycoplasma haemosuis*', and '*Candidatus Mycoplasma wenyonii*'. Int J Syst Evol Microbiol 51: 891-899.)

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Table 4.4. Erythrocyte inclusions other than organisms: Identifying features, clinical significance, and associated pathogenic processes

Inclusions	Identifying features[a]	Clinical significance	Associated pathogenic processes
Basophilic stippling	Fine to coarse, blue to dark purple dots or specks that represent aggregated ribosomes dispersed in an erythrocyte's cytoplasm (Plate 2.*Q*)	Regenerative anemia (especially cattle), plumbism	Young cells—persistence of ribosomal RNA; plumbism—inhibition of pyrimidine 5′-nucleotidase
Distemper inclusion in dogs	Round or variably shaped, pale blue or azurophilic, homogeneous inclusion; variable sizes (< 0.3–3.0 μm) (Plate 2.*R* and *S*)	Indicates active distemper infection; rare finding	—
Heinz body	Slightly pale round structure that creates a membrane defect, protrudes from erythrocyte, or occurs as free body; stains blue with NMB stain (Plate 2.*T* and *U*)	Exposure to oxidants	Oxidants overwhelm reductive capacity of erythrocyte; hemoglobin precipitates and may bind with erythrocyte membrane.
Hemoglobin crystal	Intensely stained, crystallized hemoglobin that forms a pencil, parallelogram, cube or other polyhedron within erythrocytes (Plate 2.*V*)	None in domestic mammals; most frequent in cats	Occurs with hemoglobinopathies in people
[b]Howell-Jolly body	Usually a homogenous, dark purple staining, round structure in erythrocytes; not associated with membrane; can be ring forms (especially in cats) (Plate 2.*W* and *X*)	Increased erythropoiesis, decreased splenic function	Nuclear remnant that remained free in the cytoplasm after mitosis; persists in erythrocyte if spleen does not pit it
Siderotic granule	Loose aggregate of fine granular basophilic inclusion; stain blue with Fe stains (Mallory, Prussian blue) (Plate 3.*A*)	Excess Fe in body; plumbism in dogs; MPD	Fe accumulates in damaged mitochondria or in autophagocytic vacuoles.

[a] Appearance as seen on a Wright-stained blood film unless stated otherwise.

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3. Except in cats, the presence of Heinz bodies in an animal with a hemolytic anemia indicates Heinz body hemolysis. Heinz bodies can be found in cats without clinical anemia or hemolysis.

D. Hemoglobin crystals (see Plate 2.*V*)
1. Hemoglobin crystals are seen occasionally in domestic mammal erythrocytes (including dogs and cats) but their significance is unknown. Some may form *in vitro* because of sample storage conditions.
2. Hemoglobin electrophoresis has failed to demonstrate abnormal Hgb molecules in domestic mammals.

E. Howell-Jolly body (see Plate 2.*W* and *X*)
1. A Howell-Jolly body is a nuclear remnant that remained free in the cytoplasm after mitosis of an erythrocyte precursor (it was not incorporated into a new nucleus).
2. Howell-Jolly bodies can be found in healthy mammals, frequently in cats and occasionally in dogs and horses. Increased frequency of Howell-Jolly bodies occurs during accelerated erythropoiesis and also may be found in mammals with decreased splenic function (including after splenectomy).

F. Siderotic granules (Pappenheimer bodies) (see Plate 3.*A*)
1. It may be difficult to differentiate siderotic granules from basophilic stippling on Wright-stained blood films. Siderotic granules tend to occur as loose basophilic aggregates. Basophilic stippling tends to be dispersed throughout an erythrocyte's cytoplasm. Confirmation of siderotic granules requires identification of positive-blue staining of granules with a Prussian blue or Mallory stain.
2. A siderocyte is a non-nucleated erythroid cell (reticulocyte or mature erythrocyte) that contains one or more siderotic granules. If the siderotic granules are in a nucleated erythrocyte, the cell is a sideroblast.
3. A transient siderocytosis has been associated with chloramphenicol therapy in dogs. Siderocytosis and sideroblastosis may be related to Fe overload.

V. Abnormal erythrocyte size (volume)

A. Erythrocytes appear two-dimensional on a Wright-stained blood film and thus a cell's diameter is frequently considered to reflect its size. However, it is important to recognize that two cells with the same diameter but with different thickness have different volumes. An erythrocyte's thickness is reflected by the cell's staining intensity; a thin cell will be lightly stained (hypochromic) whereas a thick cell will be stained more intensely (hyperchromic).

B. Anisocytosis: variation in diameters or volumes of erythrocytes
1. Anisocytosis can be due to macrocytes, microcytes, or both. Because of their decreased diameters, spherocytes may produce visual anisocytosis even if the spherocyte volumes are not decreased.
2. Its diagnostic significance depends on the cells that are creating the anisocytosis; it is commonly associated with macrocytosis and thus regenerative anemias.

C. Macrocyte: erythrocyte that has increased volume (see Plate 3.*B*)
1. Macrocytosis is an increased number of macrocytes in peripheral blood, which can be reflected by an increased MCV.
2. Macrocytosis is frequently seen with accelerated erythropoiesis, because immature erythrocytes are typically larger than mature erythrocytes. Macrocytes may result from a skipped mitosis in disorders of abnormal erythropoiesis.

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Table 4.5. Abnormally shaped erythrocytes (poikilocytes): Identifying features, clinical significance, and pathogeneses in domestic mammals

Poikilocyte	Other name	Identifying features	Clinical significance	Pathogenesis
Acanthocyte (*acantho* = spur)	Spur cell, burr cell[a]	2–20 irregularly spaced, membrane projections of variable lengths; projections may be blunt spurs or clubs (Plate 3.*E* and *T*).	Hemangiosarcoma; occasionally splenic, hepatic, and renal disorders	Unknown in domestic mammals; can form from changes in membrane lipids
[b]Codocyte (*codo* = hat)	Target cell, Mexican hat cell	Central focus of Hgb that is surrounded by a ring of pallor that separates it from peripheral Hgb; one form of leptocyte (Plate 3.*F*.)	Typical with regenerative anemias; also seen with hepatic, renal, and lipid disorders	Excess membrane relative to Hgb content; may occur with membrane lipid changes
Dacrocyte (*dacro* = tear)	—	Teardrop shaped (Plate 3.*G* and *H*)	Marrow diseases such as myelofibrosis and neoplasia; also may be an artifact	Unknown except artifacts caused by stretching during film preparation
Eccentrocyte (*eccentro* = eccentric)	Bite cell, cross-bonded cells, hemighost	Eccentric dense-staining Hgb and adjacent clear space or crescent (Plate 3.*I*)	Overwhelming exposure to oxidants; also rare cases of G6PD or FAD deficiencies	Fusion of membranes damaged by oxidants
[b]Echinocyte (*echino* = spiny)	Burr cell[a]	Vary from irregularly shaped cells (Type I) to regularly spaced blunt projections (Type II) to regularly spaced pointed projections (Type III) (Plate 3.*J*)	Hyponatremic dehydration, doxorubricin toxicosis, anionic drugs	Unknown
	Crenated erythrocyte		Crenated cells are artifacts	Prolonged exposure to alkaline glass while drying
[b]Keratocyte (*kerato* = horn)	Helmet cell	Notched, flattened margin between two membrane projections (horns); variant has one horn (Plate 3.*K* and *L*)	Vasculitis, intravascular coagulation, hemangiosarcoma, caval syndrome, endocarditis	Trauma caused by impact against rigid structures or by rheologic forces
Leptocyte (*lepto* = thin)	—	Thin cell that appears as a hypochromic cell with increased central pallor (Plate 3.*M*)	Fe deficiency	Incomplete hemoglobin synthesis

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Ovalocyte (*ovalo* = egg)	Elliptocyte	Elliptical or oval cell (Plate 3.*N*)	Hereditary elliptocytosis in dogs, myelofibrosis, idiopathic in cats	Abnormal membrane proteins in hereditary form, otherwise unknown
Pincered cell	—	Button or knob joined to rest of cell by a pinched area (Plate 3.*O*)	PK deficiency, intravascular trauma	Unknown
Pyknocyte (*pykno* = condensed)	Irregularly contracted cell	Spheroid erythrocyte with condensed or contracted Hgb and perhaps small tags of fragmented membrane (Plate 3.*P* and *Q*)	Overwhelming exposure to oxidants; also rare cases of G6PD or FAD deficiencies	Unclear; may form from eccentrocytes
[a]Schizocyte (*schizo* = cut)	RBC fragment, schistocyte	Triangular, comma-shaped, small round, or irregularly shaped piece of an erythrocyte (Plate 3.*R*)	Intravascular coagulation, vasculitis, hemangiosarcoma, caval syndrome, endocarditis	Same as keratocyte
[a]Spherocyte (*sphero* = round)	—	Decreased central pallor, decreased cell diameter, increased Hgb staining intensity, and smooth margins (Plate 3.*S* and *T*)	Immune hemolysis, fragmentation hemolysis	Membrane loss due to action of macrophages or trauma
Stomatocyte (*stomato* = mouth)	—	Elongated (slit-like or mouth-like) area of cytoplasmic pallor (Plate 3.*U*)	Young erythrocytes or hereditary stomatocytosis of Alaskan malamutes	Folding of excess membrane
Torocyte (*toro* = donut-shaped)	—	Punched-out, central clear space that creates a donut-shaped cell (Plate 3.*V*)	None; do not confuse with hypochromia	Artifact

[a] Classifying cells as burr cells is not recommended because the name is used for acanthocytes and echinocytes.

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3. Poodles with hereditary poodle marrow dyscrasia have uniform normochromic macrocytes (MCV: 85–95 fL).

D. Microcyte: erythrocyte that has decreased volume (see Plate 3.*C* and *D*)
   1. Microcytosis is an increased number of microcytes in peripheral blood, which can be reflected by a decreased MCV.
   2. Causes of microcytosis include Fe deficiency (usually see concurrent hypochromasia) and hepatic failure in dogs (especially due to portosystemic shunts).
   3. As seen with Fe deficiency, microcytes are created by increased cell divisions of erythroid cell precursors while they were attempting to produce their optimal [Hgb]. A microcyte can have a normal diameter but have increased central pallor due to its thinness (a hypochromic microcyte).
   4. Dogs in some breeds (e.g., Akita, Shiba) may have erythrocytes whose MCV values are 50–60 fL though most breeds have MCV values of 60–77 fL.[3] Young horses (up to 6 months of age) have lower MCV values than mature horses.[4] Young kittens have lower MCV values than mature cats.[5]
   5. Spherocytes may appear microcytic because of decreased diameters when they typically have MCV values WRI.

VI. Abnormal erythrocyte shape (Table 4.5)

A. Acanthocyte (spur cell and burr cell) (see Plate 3.*E* and *T*)
   1. Acanthocytes are most common in dogs. In dogs, acanthocytosis is associated with splenic and hepatic disorders and especially splenic hemangiosarcoma. Why acanthocytes form in these disorders is not known.
   2. In people, acanthocytic change is considered the result of abnormal lipid composition (high cholesterol to phospholipid ratio) acquired within an erythrocyte's membrane during circulation.

B. Blister cell (see keratocyte and eccentrocyte below)

C. Burr cell: common name for many spiculated erythrocytes
   1. Crenated erythrocytes, echinocytes, acanthocytes, and other spiculated erythrocytes (those with membrane projections) are called "burr cells" by different people.
   2. Because the term may refer to several types of poikilocytes, its use may lead to confusion and thus its value is limited.

D. Codocyte (target cell, Mexican hat cell) (see Plate 3.*F*)
   1. A codocyte's shape results from a central bulge in the cell due to an increased ratio of cell membrane to Hgb content.
   2. Codocytosis is commonly seen in regenerative anemias because young erythrocytes have excess membrane and decreased [Hgb]. When not associated with a regenerative anemia, codocytosis is seen in hypochromic states (e.g., Fe deficiency) and when erythrocytes have excess membrane (e.g., hepatic, renal, and lipid metabolism disorders).

E. Crenated erythrocytes (see echinocyte)

F. Dacrocyte (see Plate 3.*G* and *H*)
   1. Dacrocytosis is occasionally seen in animals with marrow diseases such as myelofibrosis and neoplasia.
   2. Artifactual dacrocytes may form because of erythrocyte stretching during blood film preparation. Artifactual dacrocytes tend to have sharp points, occur in streaks, and tend to point the same direction because directional forces of slide preparation create them.

G. Eccentrocyte (bite cell, cross-bonded cell, hemighost cell) (see Plate 3.*I*)

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1. Eccentrocytes form when oxidation leads to a bonding of erythrocyte membranes and results in a collapsed, peripheral, crescent-shaped region of the cell (sometimes called a blister) and the cell's Hgb is displaced eccentrically.
2. The membranes are damaged by some of the same oxidants that cause Heinz body anemias.
3. Eccentrocytes may form when reducing pathways in erythrocytes are defective(e.g., in G6PD and FAD deficiency in horses).

H. Echinocyte (burr cell) (see Plate 3.*J*)
1. The number and shape of spicules classify echinocytes: Type I (irregular or angular cells lacking distinct spicules), Type II (multiple regularly spaced blunt spicules), and Type III (multiple regularly spaced sharp projections).[6,7] This classification is similar to stage 1, stage 2, and stage 3 echinocytic changes seen with electron microscopy.[8]
2. Artifactual echinocytes are called crenated erythrocytes and usually are Type II or III echinocytes. Clinically, an echinocyte is often considered a crenated erythrocyte until proven otherwise. Crenation occurs after blood is collected and frequently during blood film drying. Erythrocytes of some sick animals are more prone to crenation.
3. Pathologic echinocytosis has been associated with several disorders.
   a. Erythrocyte dehydration (especially with hyponatremia and hypochloremia in horses)[6,9]
   b. Strenuous exercise (racing horses)[7]
   c. Doxorubicin toxicosis[10]
   d. Reaction to anionic drugs such as phenothiazine[11]
   e. PK deficiency (echinocytes or spheroechinocytes seen in some canine cases)[12,13]
   f. Rattlesnake envenomation[14]

I. Elliptocyte (see ovalocyte)

J. Keratocyte (helmet cell) (see Plate 3.*K* and *L*)
1. Keratocytosis may be caused by trauma to erythrocytes within the vascular system; the same processes may create schizocytes.
2. An intermediate form sometimes called a prekeratocyte (blister cell) has a bulging cytoplasmic clear space (blister) that may represent a vacuole.

K. Leptocyte (see Plate 3.*M*)
1. Some codocytes and most hypochromic erythrocytes are leptocytes (see codocytosis and hypochromasia for clinical significance).
2. Some people consider *leptocyte* a synonym for *codocyte* or a term for a hypochromic erythrocyte. Codocytes and hypochromic erythrocytes may be leptocytes, but not all leptocytes are codocytes or hypochromic erythrocytes. Also, immature erythrocytes may be hypochromic or codocytes without being leptocytes.

L. Ovalocyte (elliptocyte) (see Plate 3.*N*)
1. Ovalocytosis is seen in dogs with myelofibrosis; it is also seen in dogs that have an abnormal structural protein in the erythrocyte's membrane (probably a congenital disorder), and in animals with Fe deficiency anemias (along with other abnormalities). It is occasionally seen in feline blood films but the cause or significance is not known.
2. Normal shape for llama, other camelid, avian, reptilian, and amphibian erythrocytes

M. Pincered cell (see Plate 3.*O*)
1. Has been associated with erythrocyte trauma and PK deficiency in a Cairn terrier (unpublished case report)

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2. In people, pincered cells have been associated with erythrocyte fragmentation and hereditary spherocytosis.[15]

N. Poikilocyte
1. A poikilocyte is an erythrocyte with an abnormal shape. Poikilocytosis is an increased number of poikilocytes in blood. The significance of poikilocytosis depends on the type of poikilocyte present; poikilocytes can represent artifacts or pathologic cells.
2. Erythrocytes of neonatal calves (especially if anemic) can have spiculated erythrocytes with features of acanthocytes, echinocytes, or schizocytes. The pathogenesis of the changes is not established but may be related to presence of a unique hemoglobin molecule or its interactions with erythrocyte membrane proteins.[16] Iron deficiency may also contribute to the anemia and poikilocytosis.[16,17]

O. Pyknocyte (irregularly contracted cells) (see Plate 3.*P* and *Q*)
1. Pyknocytosis is seen concurrently with eccentrocytosis in dogs and horses and probably will be seen in other animals. Pyknocytes may form from eccentrocytes or oxidative damage might cause both directly.
2. Pyknocytes stain more intensely with NMB stain than do discocytes or spherocytes.
3. On stained blood films, some pyknocytes look like spherocytes. However, via electron microscopy, pyknocytes had membrane irregularities or tags and were not perfect spheres.[18]

P. Schizocyte (schistocyte or RBC fragment) (see Plate 3.*R*)
1. Schizocytosis occurs when rigid structures or rheologic forces traumatize erythrocytes.
2. Pathologic states associated with schizocytosis include intravascular coagulation, vasculitis, hemangiosarcoma, caval syndrome of dirofilariasis, and endocarditis.

Q. Spherocyte (see Plate 3.*S* and *T*)
1. For domestic mammals, spherocytes are most easily recognized in canine blood films but can be recognized or suspected in other species with careful evaluation.
2. Its spheroid shape usually results from the loss of erythrocyte membrane without a corresponding loss in erythrocyte volume. Spherocytes are frequently seen in immune hemolytic anemias and may be seen in fragmentation anemias along with other poikilocytes. Spherocytes induced by fragmentation may have decreased volume (microspherocytes). They also are reported to occur in PK-deficient dogs[19] and in bee-sting anemias.[20,21]

R. Stomatocyte (see Plate 3.*U*)
1. Stomatocytes result from folding of excess membrane to form an elongated area of pallor instead of circular central pallor. Young erythrocytes (polychromatophilic erythrocytes or young macrocytes) frequently are stomatocytes.
2. Stomatocytosis also results from a hereditary defect in erythrocyte membrane (hereditary stomatocytosis of Alaskan malamutes, miniature schnauzers, and Drentse patrijshond).[22,23]

S. Torocyte (see Plate 3.*V*)
1. Represents an artifactual shape change
2. Torocytes should not be confused with hypochromic cells that have marked central pallor. A torocyte has a sharply punched out center and a dense ring of Hgb staining in its periphery; a hypochromic cell has a paler ring of peripheral Hgb staining that fades into central pallor.

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ANEMIA

I. General information

A. Definition: a decreased [RBC], a decreased [Hgb], or a decreased Hct of peripheral blood

B. A blood's Hct, [Hgb], and [RBC] generally change proportionately because they all assess the erythrocyte content of blood. However, they may not be uniformly decreased because of variations in reference intervals or the presence of abnormal erythrocytes (abnormal erythrocyte volume or intracellular Hgb concentration).

C. Anemia is a pathologic state or diagnostic problem; it is not a disease. Its major significance is reduced capacity of blood to transport O_2 to tissues. Anemia develops when there is one or both of the following:

1. Increased erythrocyte loss due to hemolysis or blood loss
2. Decreased or ineffective erythrocyte production

D. Clinical signs caused by anemia reflect decreased O_2-carrying capacity and include decreased exercise tolerance, weakness, depression, and rapid respiration (tachypnea).

E. The major physical examination finding is pale mucous membranes (gingival, conjunctival, vulvar) due to dilute blood in capillaries. With a marked anemia, blood becomes less viscous and may cause a systolic heart murmur.

II. Classifications of anemias

There are three common classification systems; each has its advantages and limitations in certain clinical situations.

A. Classification by marrow responsiveness

1. This classification system is primarily based on the presence or absence of reticulocytosis in blood; other blood film and marrow findings may influence the classification.
   a. Regenerative anemia = anemia with a concurrent reticulocytosis
   b. Nonregenerative anemia = anemia without a concurrent reticulocytosis
2. Reticulocytosis is typically established by finding an increased RC, increased CRP, or increased polychromasia (see Chap. 2).
   a. Documenting reticulocytosis is the most reliable, single routine method of establishing accelerated erythropoiesis (except in horses).
   b. In most species, reticulocytosis is expected about 3–4 days after Epo stimulates marrow; peak production is expected about 7–10 days after stimulation.
   c. Animals in each species vary in their ability to produce a reticulocytosis. Dogs have the greatest ability; RC or CRP may increase sixfold to eightfold in response to severe anemia. Cats have moderate ability (maybe threefold to fivefold). Cattle have mild ability; increased polychromasia is frequently accompanied by erythrocytes with basophilic stippling. Horses very rarely release reticulocytes from marrow and thus attempting to establish peripheral blood reticulocytosis is not valuable. Macrocytes in equine blood suggest, but do not prove, marrow responsiveness to Epo.
3. The following erythrocyte abnormalities would support a regenerative status but each may also be found in nonregenerative anemias: macrocytic and/or hypochromic indices, anisocytosis, Howell-Jolly bodies, rubricytosis, codocytosis, basophilic stippling.
4. Bone marrow erythroid hyperplasia without a reticulocytosis may reflect a pending regenerative anemia but also could represent ineffective erythropoiesis.

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5. A progressively increasing Hct, even in the absence of a reticulocytosis, indicates a responsive marrow and a regenerative anemia. This may occur in horses or in other animals that have mild anemias or that are in the resolving stages of more severe anemias.
6. Marrow responsiveness classifications
    a. Regenerative anemia (responsive anemia)
        (1) Seen primarily in response to blood loss or hemolysis; rarely associated with erythroid neoplasia in cats
        (2) Regenerative status indicates that a bone marrow is regenerating a replacement population of erythrocytes.
    b. Nonregenerative anemia (nonresponsive anemia)
        (1) Seen in diseases that directly or indirectly cause defective or reduced erythrocyte 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.)
        (2) A nonregenerative status indicates that bone marrow is not regenerating a replacement population of erythrocytes. A severe, nonregenerative anemia typically reflects severe and prolonged damage to erythroid cell precursors.
        (3) Findings in bone marrow examinations include erythroid hypoplasia, marrow aplasia, red cell aplasia, myelofibrosis, myelitis, myelophthisis, relatively normal erythroid series, or erythroid hyperplasia in early responsive anemias.
        (4) Most nonregenerative anemias are normocytic normochromic anemias without poikilocytosis or other erythrocyte abnormalities. However, blood may contain the following erythrocyte abnormalities related to the underlying disease process: Howell-Jolly bodies, rubricytosis, codocytosis, basophilic stippling, macrocytes or microcytes, hypochromic erythrocytes.

B. Morphologic classification
1. The morphologic classification system is based on MCV and MCHC values (Table 4.6); classification should be confirmed by examining erythrocytes on a Wright-stained blood film.
2. General concepts
    a. MCV and MCHC values suggest the type of erythrocyte that is being produced by the marrow.
        (1) Normocytic: erythroid cell maturation not defective
        (2) Macrocytic: young erythrocytes present or there is defective erythrocyte maturation
        (3) Microcytic: increased mitoses during erythrocyte maturation
        (4) Normochromic: Hgb synthesis complete
        (5) Hypochromic: incomplete Hgb synthesis (young erythrocytes or defective synthesis)
    b. Because MCV and MCHC are averages, a blood film examination is typically a more sensitive method of detecting macrocytic, microcytic, or hypochromic cells. It is possible to have a normocytic normochromic anemia but find macrocytosis, microcytosis, or hypochromasia in a blood film.
3. Morphologic classifications of anemias
    a. Normocytic normochromic anemias
        (1) Blood film findings: typically uniform erythrocytes; may have occasional erythrocytes with morphologic abnormalities

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Table 4.6. Morphologic classification of anemias with reported causes

Anemia classification	MCV	MCHC[a]	Disorders or conditions that cause the anemia
Normocytic normochromic	WRI	WRI	If persistent, then typically disorders that cause reduced erythropoiesis; most anemias begin as normocytic normochromic.
Macrocytic hypochromic	↑	↓	Regenerative response after blood loss or hemolysis
Macrocytic normochromic	↑	WRI	Regenerative response after blood loss or hemolysis; occasionally due to defective erythropoiesis (FeLV induced, Poodle macrocytosis); *in vitro* changes[b]
Microcytic hypochromic	↓	↓	Fe deficiency, pyridoxine deficiency
Microcytic normochromic	↓	WRI	Fe deficiency, hepatic failure including portosystemic shunts, *in vitro* changes[b]
Normocytic hypochromic	WRI	↓	Rarely seen

Note: Erythrocyte concentrations in juvenile animals typically are lower than in mature animals.

[a] Classification system does not include categories for ↑ MCHC; if there is an ↑ MCHC value, the value is usually an erroneous value (see text) and the true value may be WRI or even decreased.

[b] See text for causes of *in vitro* changes that can produce higher or lower MCV values.

(2) Most anemias begin as normocytic normochromic anemias. When marrow releases many larger or smaller erythrocytes with normal or decreased Hgb concentrations, then MCV or MCHC values will change. MCV or MCHC values must be outside of reference intervals before the morphologic classification changes.

(3) Persistent normocytic normochromic anemias are expected to be nonregenerative anemias.

(4) Most anemias in horses are normocytic normochromic because their marrows rarely release reticulocytes. If sufficient macrocytes are released, the anemia will become macrocytic.

b. Macrocytic hypochromic anemias

(1) Blood film findings: expect polychromasia (except in horses), macrocytosis, and anisocytosis; hypochromasia may not be recognized because of the basophilia of polychromatophilic erythrocytes or because of insufficient biconcavity to create central pallor.

(2) Concurrent macrocytosis and hypochromia indicate the presence of immature erythrocytes and thus the anemia is probably due to blood loss or hemolysis.

c. Macrocytic normochromic anemias

(1) Blood film findings: expect polychromasia, macrocytosis and anisocytosis

(2) Common in regenerative anemias due to blood loss or hemolysis

(3) Sometimes associated with defective erythropoiesis

(a) FeLV-infected cats may have defective erythroid maturation that yields megaloblastic cells (Plate 3.*X*) with defective DNA synthesis and thus decreased mitosis; megaloblastic cells mature to macrocytes.

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(b) Folic acid and cobalamin (vitamin B_{12}) deficiencies cause defective nucleic acid metabolism that could result in macrocytosis (possible, but rarely documented). Cattle that graze cobalt-deficient pasture may have a macrocytosis due to a cobalamin deficiency.[24,25] Cobalt is an essential component of cobalamin. Cobalamin deficiency blocks folate metabolism by trapping a methyl group in 5-methyltetrahydrofolate. Thus, in the absence of cobalamin, a functional folate deficiency may exist even though serum folate concentration may be WRI.

(c) Poodles with the poodle marrow dyscrasia will have a macrocytosis and may have an anemia due to another pathologic process. The pathogenesis of the macrocytosis is not established.

(d) Erythroleukemia

(e) Congenital dyserythropoiesis and progressive alopecia of polled Hereford calves[26]

(4) Increased MCV value may be produced by *in vitro* events or phenomena.

(a) Erythrocyte agglutination: An electronic cell counter may consider an aggregate (mostly doublets and triplets) of erythrocytes to be one large erythrocyte; agglutination could be immune-mediated or, in horses, induced by heparin.

(b) Cell swelling during storage before testing; occurs most frequently with mail-in samples; MCHC may be decreased.

(c) *In vivo* hyperosmolar states (e.g., hypernatremia) can lead to increased intracellular osmolality; when the blood within the analyzer is diluted by fluid of lower osmolality (approximately isoosmotic with normal plasma), H_2O moves into the cells and causes acute swelling; MCHC may be decreased.

(d) Excess EDTA anticoagulant may cause erythrocyte swelling (Technicon instrument)[27] when the cells mix with analyzer diluent.

d. Microcytic hypochromic anemia

(1) Blood film findings: expect microcytosis, leptocytosis, codocytosis, hypochromia, and anisocytosis; may see ovalocytes and fragmented and folded erythrocytes; polychromasia may be present but less than expected for the severity of the anemia.

(2) Microcytosis and hypochromasia may be due to defective Hgb synthesis.

(a) Fe deficiency (see Blood Loss Anemias, II.B)

(b) Copper deficiency in pigs but not in dogs; experimental Cu deficiency in dogs produced a normocytic normochromic anemia.[28]

(c) Potential vitamin B_6 (pyridoxine) deficiency

(d) Hepatic failure (rarely, more likely microcytic normochromic)

e. Microcytic normochromic anemia

(1) Blood film findings: varies from those seen in microcytic hypochromic anemia to normocytic normochromic anemia

(2) Causes of microcytosis

(a) Fe deficiency (early or mild): Prior to causing a microcytic hypochromic anemia, Fe deficiency may produce a microcytic normochromic anemia but the MCH is decreased.

(b) Hepatic failure due to hepatic disease or portosystemic shunts; cause of microcytosis not known but data suggest a defect in Fe transport to ery-

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throcyte precursors; MCH is decreased but MCHC typically remains WRI.

(c) Dyserythropoiesis of English springer spaniels[29]

(d) Some healthy dogs in the Akita and Shiba breeds have lower MCV values (in the 50–60 fL range) than do other dog breeds. Also, foals and kittens have lower MCV values than adult animals of the respective species.[4,5]

(3) Factitiously low MCV and high MCHC values may be produced when erythrocytes are in hypoosmolar plasma.[30] Erythrocytes adjust *in vivo* to the hypoosmolal environment caused by hyponatremia and hypochloremia by having decreased cytoplasmic osmolality. When put in a diluent prior to counting, osmosis results in H_2O leaving the erythrocytes and thus decreasing volume of erythrocytes. MCV may be decreased and MCHC may be increased erroneously.

f. Normocytic hypochromic: An uncommon finding; if found, one must consider that the data may be inaccurate or the reference intervals may be inappropriate.

4. Increased MCHC values
   a. In theory, it is not physiologically possible to produce hyperchromic erythrocytes because Hgb synthesis stops in an erythrocyte precursor when an optimal [Hgb] is reached within its cytoplasm.
   b. Most increased MCHC values are falsely increased and the blood sample's MCH value also is falsely increased. Causes of falsely increased MCHC and MCH values include the following:
      (1) Hemoglobinemia: Blood [Hgb] used to calculate MCHC and MCH values would include Hgb from erythrocytes and the free Hgb in plasma.
      (2) *In vitro* hemolysis: Blood [Hgb] used to calculate the MCHC would truly represent the blood [Hgb] but the Hct and [RBC] values for the sample would be falsely decreased.
      (3) Spectral interferences in the blood Hgb assay: Interferences giving falsely increased [Hgb] include lipid droplets in grossly lipemic samples, pigments in markedly icteric samples, nuclei or intact WBCs in samples with extreme leukocytosis, and Heinz bodies (incomplete erythrocyte lysis).
      (4) As discussed above, MCHC may be falsely increased because of cell shrinkage related to *in vivo* hypoosmolal states (e.g., hyponatremia) followed by cell contact with a relatively hyperosmolal diluent in the analyzer.
   c. Pathologic conditions that can cause true increases in MCHC values are rare.
      (1) Eccentrocytes and pyknocytes may have increased MCHC values because oxidative condensation of Hgb and fusion of cell membranes causes a loss of cell volume without a proportionate loss of cell Hgb.
      (2) Spherocytes with increased MCHC values may potentially form in some spherocytic anemias if the spherocytic process causes loss of cell volume in excess of Hgb; generally, however, spherocytes only *appear* to be hyperchromic because of their thickness, and their MCHC values are WRI.

C. Pathophysiologic classification
   1. Based on the pathologic mechanism or process that produced the anemia
      a. Blood loss anemias: can be acute (hours to days) to chronic (weeks to months)
         (1) External blood loss anemias: erythrocytes lost from body or lost into the alimentary or urinary tract

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(2) Internal blood loss anemias: movement of erythrocytes from intravascular to extravascular space (typically into peritoneal or pleural cavities)

b. Hemolytic anemias
   (1) Extravascular hemolysis: erythrocyte lysis outside of blood vessels (in macrophages)
   (2) Intravascular hemolysis: erythrocyte lysis within the blood vascular system
c. Anemias due to decreased erythrocyte production (see the next section, Nonregenerative Anemias, for more information)
   (1) Inflammatory diseases
   (2) Renal disease
   (3) Marrow hypoplasia or aplasia
   (4) Erythroid hypoplasia or ineffective erythropoiesis

2. The pathophysiologic classification system is frequently used in one of two ways.
   a. Serves as a differential diagnosis list or to answer questions such as "What are the basic causes of anemias?" (Fig. 4.6)
   b. To group specific diseases based on the method or methods by which they cause anemia

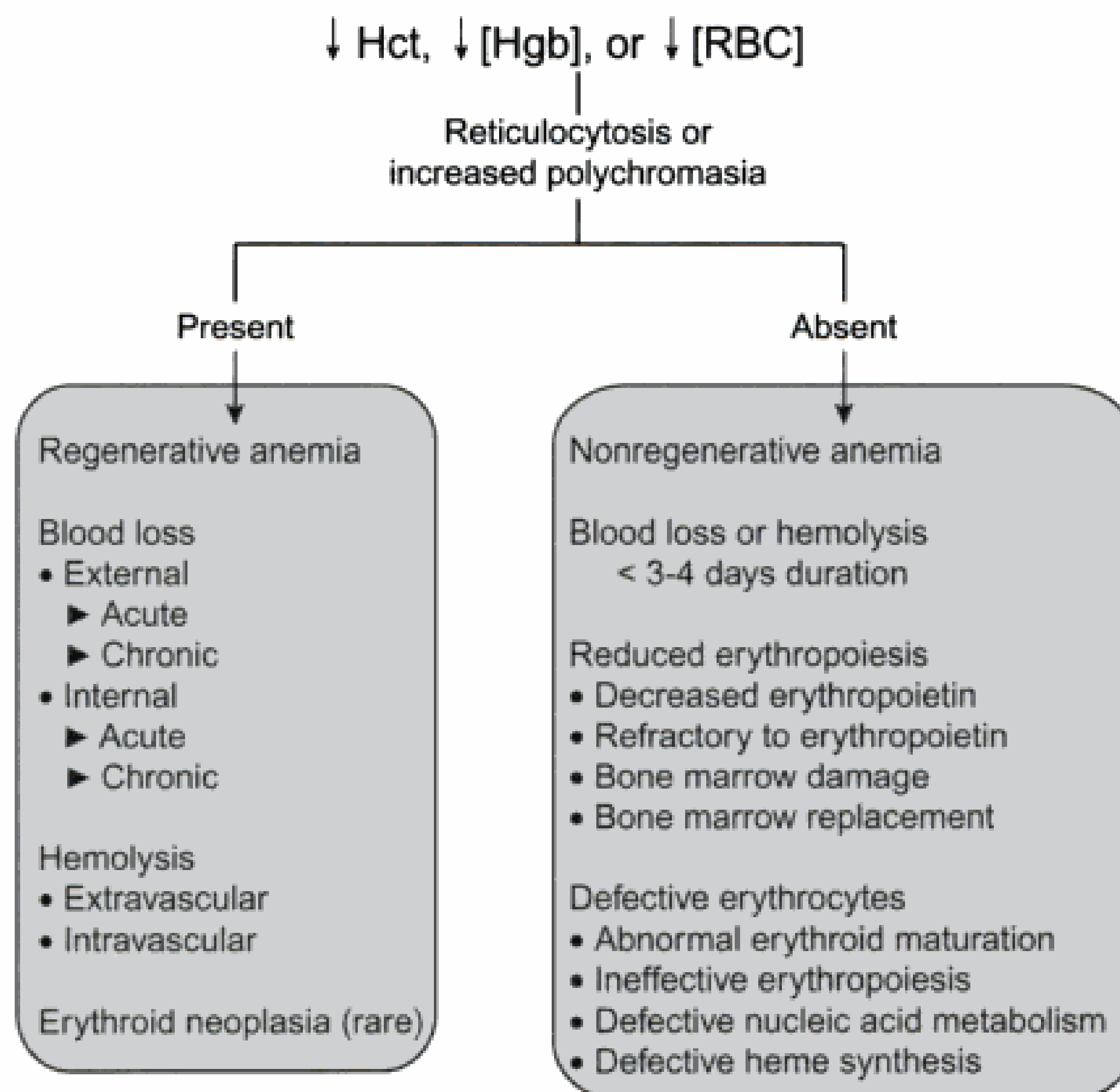

Fig. 4.6. An approach to finding the cause of anemias.
After anemia has been detected or confirmed, the presence or absence of a regenerative response is determined by assessing RC or CRP or detecting increased polychromasia (see Chap. 2). If it is a regenerative anemia, then the anemia is probably due to either blood loss or hemolysis. If it is a nonregenerative anemia and the anemia has been present for several days, then the anemia is probably due to reduced or defective erythropoiesis.

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NONREGENERATIVE ANEMIAS

I. General concepts

A. The major reason for a persistent nonregenerative anemia is decreased erythrocyte production; defective erythropoiesis can also contribute. Since erythrocyte life spans of domestic animals are generally 2–5 months, it will take several weeks to months for an anemia to develop if it is only due to decreased erythropoiesis. For example, a dog's erythrocyte life span is about 100 days. In health, 50% of its erythrocytes are > 50 days old and 50% are < 50 days old. If a disease stopped erythropoiesis completely and did not alter erythrocyte life span, it would take 25 days for the dog's Hct to drop from 40% to 30%, and about 50 days to drop from 40% to 20%. Because cat erythrocytes have shorter life spans (about 70 days), such an anemia would develop quicker. Likewise, production-failure anemia would develop slower in horses and cattle because their erythrocytes have longer life spans.

B. Most diseases do not completely stop erythrocyte production; they only decrease the rate of production. Therefore, nonregenerative anemias may take even longer to develop. However, many diseases that reduce erythropoiesis also cause a shortened erythrocyte life span and thus anemia may develop quicker than expected from reduced erythropoiesis alone.

C. Most animals with nonregenerative anemias have been anemic for several weeks before clinical signs are detected. Because the anemia is chronic, the disease or disorder causing the anemia is chronic. Severity of a nonregenerative anemia will depend on duration of the disease process, degree of decreased erythropoiesis, and presence of other processes that shorten erythrocyte life span.

D. When a persistent nonregenerative anemia is detected, most erythrocytes in the animal's blood were produced when the animal had the disease that caused the anemia. Thus, characteristics of the circulating erythrocytes may help determine the cause of the persistent nonregenerative anemia (see morphologic classification in Anemia, II.B above).

II. Disorders that cause nonregenerative anemias (Table 4.7)

A. Inflammatory disease

Table 4.7. Disorders and conditions that cause nonregenerative anemias

- Inflammatory diseases (primarily chronic)
  - [a]Infectious: bacterial, fungal, viral, protozoal, parasitic
  - [a]Noninfectious
- [a]Renal disease (chronic)
- Diseases causing marrow hypoplasia or aplasia
  - Myelitis: bacterial, fungal, viral, protozoal
  - Toxicosis: chemotherapeutic agents, estrogen, bracken fern, phenylbutazone
  - Irradiation: whole-body or environmental
  - Marrow replacement: neoplasia, myelofibrosis, osteopetrosis
- Diseases causing erythroid hypoplasia or ineffective erythropoiesis (without generalized marrow hypoplasia)
  - Pure red cell aplasia
  - [a]FeLV-induced erythroid hypoplasia
  - Nutritional: Fe, copper, folate or vitamin B_{12} deficiency
  - [a]Endocrine: hypothyroidism, hypoadrenocorticism, hypoandrogenism
  - [a]Liver disease or failure (including portosystemic shunts)

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1. Inflammation causes an anemia that is called AID (also called anemia of chronic disease, anemia of inflammation). It is the most common nonregenerative anemia of domestic mammals and varies from mild to moderate severity. Typically, it is a normocytic normochromic anemia but, rarely, it is microcytic.
2. AID is of relatively little clinical significance after it is recognized. Most diagnostic efforts are directed toward the primary disease process and not the secondary abnormalities caused by the inflammatory disease.
3. Almost any chronic disorder with an inflammatory component will initiate the processes that cause the anemia.
   a. Chronic infections: bacterial (including rickettsial), fungal, viral, protozoal
   b. Noninfectious disorders: immune, toxic, neoplastic (usually a malignant neoplasm that causes necrosis and/or inflammation around or within the neoplasm)
4. Pathogenesis of the anemia involves three concurrent mechanisms initiated by inflammation[31]
   a. Shortened erythrocyte survival
      (1) Pathologic events are not understood entirely but are associated with increased IL-1 concentration.
      (2) Oxidant damage to erythrocyte membranes and subsequent binding of immunoglobulin molecules may accelerate the removal of erythrocytes.[32]
   b. Impaired Fe mobilization or utilization
      (1) Alterations in ferritin production and alterations in transferrin receptors lead to increased Fe storage and therefore decreased availability of iron for Hgb synthesis.
      (2) Cytokines involved in altered Fe kinetics include IL-1, INF, and TNF.
   c. Impaired erythrocyte production
      (1) Erythroid cells become refractory (nonresponsive) to increased Epo because of effects of inflammatory cytokines (IL-1, INF, TNF) on precursors.
      (2) Blunted Epo response to anemia (Epo production is increased but not as much as expected) is due to actions of IL-1, TNF, and TGF-β.
5. Laboratory findings that, when present, support the conclusion that an animal has AID:
   a. Mild to moderate normocytic normochromic anemia with little to no poikilocytosis
   b. Chronic inflammatory leukogram: mature neutrophilia, lymphocytosis, monocytosis
   c. Hyperproteinemia due to increased concentrations of gamma globulins or other inflammatory proteins
   d. Marrow contains essentially normal erythroid cells, mild to moderate granulocytic hyperplasia, possibly plasmacytosis, and abundant hemosiderin (hemosiderin is not expected in feline marrow).
   e. Hypoferremia and adequate to increased stainable-Fe in tissues (marrow, spleen, or liver)

B. Renal disease (chronic)
   1. Most patients with chronic renal disease are anemic; anemias are slight to moderate in severity and essentially all are normocytic normochromic.
   2. Pathogenesis of anemia
      a. Inadequate Epo production: Chronic renal disease causes sufficient damage to kidneys so that Epo production is decreased and thus there is inadequate stimulation of erythrocyte production (Epo therapy is effective).

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b. Decreased erythrocyte life span (mild): Agents not cleared by the kidneys cause decreased erythrocyte life span.
c. Decreased marrow response to Epo
d. Other factors: hemorrhage due to uremic ulcers or vascular damage; poor nutritional status
3. Laboratory findings
a. Normocytic normochromic, nonregenerative anemia
b. Evidence of chronic renal disease or dysfunction, such as azotemia, urine specific gravity values in isosthenuric range, and electrolyte disturbances
C. Diseases causing marrow hypoplasia or aplasia of multiple cell lineages
1. Major concepts
a. Damage can be to one or more of the components of the marrow's microenvironment: blood vessels and/or sinusoids, reticular adventitial cells, marrow stroma (fat cells, fibrocytes), or hematopoietic stem cells. The resulting marrow will be hypoplastic or aplastic.
b. Damage may be irreversible or reversible and may result in aplastic anemia (hypoplastic pancytopenia).
2. Disorders
a. Myelitis resulting from bacterial septicemias, disseminated mycoses (e.g., histoplasmosis), viral infections (e.g., EIAV infection), or protozoal infections (e.g., leishmaniasis)
b. Toxicoses involving compounds such as chemotherapeutic agents, estrogen,[33,34] phenylbutazone,[35,36] and chemicals in bracken fern
c. Irradiation damage resulting from either whole body therapeutic or environmental exposure to X-rays, gamma, or beta irradiation
d. Marrow replacement
(1) Diseases may cause anemia by replacing hematopoietic cells in the marrow; such anemias are commonly called myelophthisic anemias.
(2) Disorders that may cause myelophthisis (*myelo-* marrow; *-phthisis* wasting)
(a) Myeloproliferative diseases: granulocytic, monocytic, erythroid, or megakaryocytic neoplasia
(b) Lymphoproliferative diseases: lymphoid and plasma cell neoplasia
(c) Metastatic neoplasia
(i) Lymphoproliferative disease (primary in lymph nodes, spleen or other tissues)
(ii) Mast cell neoplasia
(iii) Carcinomas and nonhemic sarcomas can metastasize to marrow, but such lesions are not expected to cause sufficient marrow damage to produce anemia.
(d) Non-neoplastic cell proliferation
(i) Myelofibrosis: Fibrous tissue proliferation after inflammation and/or necrosis (myelofibrosis may be a myeloproliferative disease in some cases).
(ii) Osteopetrosis: bone proliferation into medullary space
D. Diseases causing erythroid hypoplasia or ineffective erythropoiesis (without generalized marrow hypoplasia)
1. Pure red cell aplasia
a. Pure red cell aplasia is a descriptive term for disorders in which a nonregenerative

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anemia is caused by erythroid hypoplasia or aplasia, but other hematopoietic cell lines are not defective. It has been recognized in dogs,[37] cats, and people.

b. Pathogenesis of the anemia
  (1) In people, there is often either a viral infection of erythroid cells or an antibody or T-cell mediated destruction of erythroid precursors. Antibodies may even be directed against Epo.
  (2) Some dogs have had a demonstrable serum substance (probably antibody) that inhibits erythropoiesis *in vitro*, but other dogs have not.[38]

c. The disorder may be responsive to immune-suppressive dosages of glucocorticoid compounds or other immunosuppressive therapy; may take therapy 2 weeks or longer before there is evidence of response (e.g., reticulocytosis). Some dogs require long-term therapy to prevent recurrence of an anemia.

d. Laboratory findings
  (1) Typically normocytic normochromic anemia; sometimes spherocytic
  (2) Nonregenerative
  (3) May or may not be Coombs' test positive
  (4) Marrow examination reveals marked hypoplasia or aplasia (absence) of the erythroid cell lineage, with preservation of the other lineages.

2. FeLV-induced erythroid hypoplasia
  a. FeLV may selectively damage erythroid cells to cause erythroid hypoplasia or transform a cell into a neoplastic cell line.
  b. Pathogenesis of anemia
    (1) If there is selective damage to erythroid precursors, then erythroid hypoplasia or aplasia develops and thus there is decreased erythrocyte production.
    (2) If neoplastic transformation of erythroid cells occurs, there may be a marked proliferation of cells but they have defective function, cell metabolism, and maturation. Accordingly, the cells die before maturing to erythrocytes and thus anemia develops because of decreased effective erythropoiesis.
  c. Laboratory findings
    (1) Mild to severe, nonregenerative anemia; either normocytic normochromic or macrocytic normochromic
    (2) May have inappropriate rubricytosis, especially in MDS-Er
    (3) Marrow findings may vary from erythroid hypoplasia to neoplasia of any marrow cell lineage
    (4) Megaloblastic erythroid cells may be found in blood or marrow (megaloblastic anemia) (see Plate 3.X). Megaloblastic cells have asynchronous maturation of nuclei and cytoplasms; cytoplasms mature but nuclear maturation is incomplete. The defective maturation produces larger erythroid precursors with atypically large nuclei for the degree of cytoplasmic maturation.

3. Nutrient deficiencies
  a. Fe deficiency
    (1) Fe deficiency occurs because of chronic external blood loss (e.g., alimentary tract blood loss due to ulcers or parasites or cutaneous blood loss due to fleas or ticks) or inadequate dietary Fe intake (especially in neonates).
    (2) When diagnosed, the anemia is classically microcytic hypochromic but may be microcytic normochromic (for pathogenesis, see Blood Loss Anemias, II.B below).
  b. Copper deficiency

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(1) Copper deficiency is an uncommon disorder in domestic mammals but has been reported in pigs (naturally) and in dogs (experimentally).

(2) Associated with microcytic hypochromic anemias in pigs

(a) Ceruloplasmin (synonym: ferric oxidase) is a copper-containing enzyme that promotes the conversion of Fe^{3+} to Fe^{2+} for Fe transport from macrophages and into erythroid precursors. Fe^{2+} is then combined with protoporphyrin IX to form heme.

(b) If deficient in copper, there is less ferric oxidase activity, less Fe^{2+} for heme synthesis, and thus defective Hgb synthesis. Because of the copper deficiency, there is a functional Fe deficiency and a pig may develop a microcytic hypochromic anemia. Serum Fe concentrations should not be decreased.

(3) In an experimental study, copper-deficient dogs developed a normocytic normochromic anemia.[28] The pathogenesis of the anemia was not established.

c. Folate or cobalamin (vitamin B_{12}) deficiency

(1) Folate and cobalamin are required for DNA synthesis and thus deficiencies might cause abnormal erythrocyte development. Folate and cobalamin deficiencies may cause a macrocytic anemia in people but rarely are such disorders found in domestic mammals.

(2) Cats with experimental folate deficiency had megaloblastic marrow erythroid cells but neither macrocytosis nor anemia.[39] A cat with a congenital cobalamin deficiency had normocytic erythrocytes.[40]

(3) Giant schnauzers with an inherited malabsorption of cobalt had a cobalamin deficiency and a normocytic anemia. Marrow samples contained megaloblastic erythroid cells, and macrocytes and ovalocytes were found in blood films. Reportedly, an increased MCV was not present because of concurrent microcytosis; an explanation of the microcytosis was not provided.[41]

4. Endocrine disorders

a. Hypothyroidism

(1) Seen primarily in dogs; causes a mild normocytic normochromic anemia

(2) Pathogenesis of anemia: Decreased [tT_4] and [tT_3] result in a decreased metabolic rate and thus decreased need for O_2 in peripheral tissues. Decreased need for O_2 leads to decreased Epo production and thus less erythrocyte production. A new homeostasis develops in which metabolic needs for O_2 are met by a lower blood erythrocyte concentration.

(3) Laboratory findings

(a) Mild normocytic normochromic, nonregenerative anemia

(b) Evidence of thyroid dysfunction, such as decreased [tT_4], decreased [fT_4], and increased [TSH]

b. Hypoadrenocorticism

(1) Seen primarily in dogs and may cause a mild to moderate normocytic normochromic anemia

(2) Pathogenesis of 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 may enhance the anemia.

(3) Laboratory findings

(a) Mild normocytic normochromic anemia (anemia may be masked by hemoconcentration caused by hypovolemia)

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(b) Evidence of adrenal dysfunction, such as hyponatremia, hyperkalemia, azotemia, hypocortisolemia, lymphocytosis, and eosinophilia

c. Hyperestrogenism
(1) Increased blood estrogen concentrations may result from excessive production by neoplasms (testicular and ovarian neoplasms) or from administration of estrogen compounds.
(2) Besides developing clinical signs of feminization, manifestations of hyperestrogenism in mammals (especially dogs and ferrets) may include a severe nonregenerative anemia as part of the pancytopenia of estrogen toxicosis.

5. Liver disease or insufficiency (including portosystemic shunts)
a. Mammals with chronic and usually progressive liver disease or portosystemic shunts frequently have mild to moderate anemia. Typically, the anemias are normocytic normochromic. When the disease causes hepatic insufficiency in dogs, some will have a microcytic normochromic anemia.
b. Pathogenesis of anemia
(1) The normocytic normochromic anemia could be an AID in some cases.
(2) Other potential mechanisms include defective amino acid and protein synthesis, abnormal lipid metabolism (affect erythrocyte lipid content and life span), and decreased production of an Epo precursor by hepatocytes (in some species).
c. In dogs with hepatic insufficiency, the microcytosis is not due to total body Fe deficiency. However, defective protein synthesis may create a functional Fe deficiency because of defective Fe transport.
d. Laboratory findings
(1) Mild to moderate normocytic or microcytic normochromic (or rarely hypochromic) anemia
(2) Evidence of liver disease (e.g., increased serum hepatic enzyme activities) or hepatic dysfunction (e.g., decreased serum urea concentration, hypoproteinemia, hypoalbuminemia, increased serum bile acid concentration, hyperammonemia, ammonium biurate crystalluria)

BLOOD LOSS ANEMIAS

I. Causes of blood loss
A. Hemorrhage
1. Blood vessels damaged by trauma, ulceration, neoplasia, or other means
2. Acquired or congenital coagulation factor deficiencies or von Willebrand disease
3. Thrombocytopenia (marked)
B. Parasitism: hookworms and whipworms (dog), haemonchosis and ostertagiosis (ruminant), coccidiosis, ticks, blood-sucking lice, and fleas (dog, cat, and calves[42])
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. Acute blood loss occurs when blood is lost from the vessels in a few hours. Anemia results from the dilution of erythrocytes that remain in vessels (Fig. 4.7).
a. If anemia is due to hemothorax or hemoperitoneum, severity of the anemia may be diminished by resorption of about 65% of erythrocytes within 2 days and 80% within 1–2 weeks.[43] Also, animals do not become Fe-depleted because ery-

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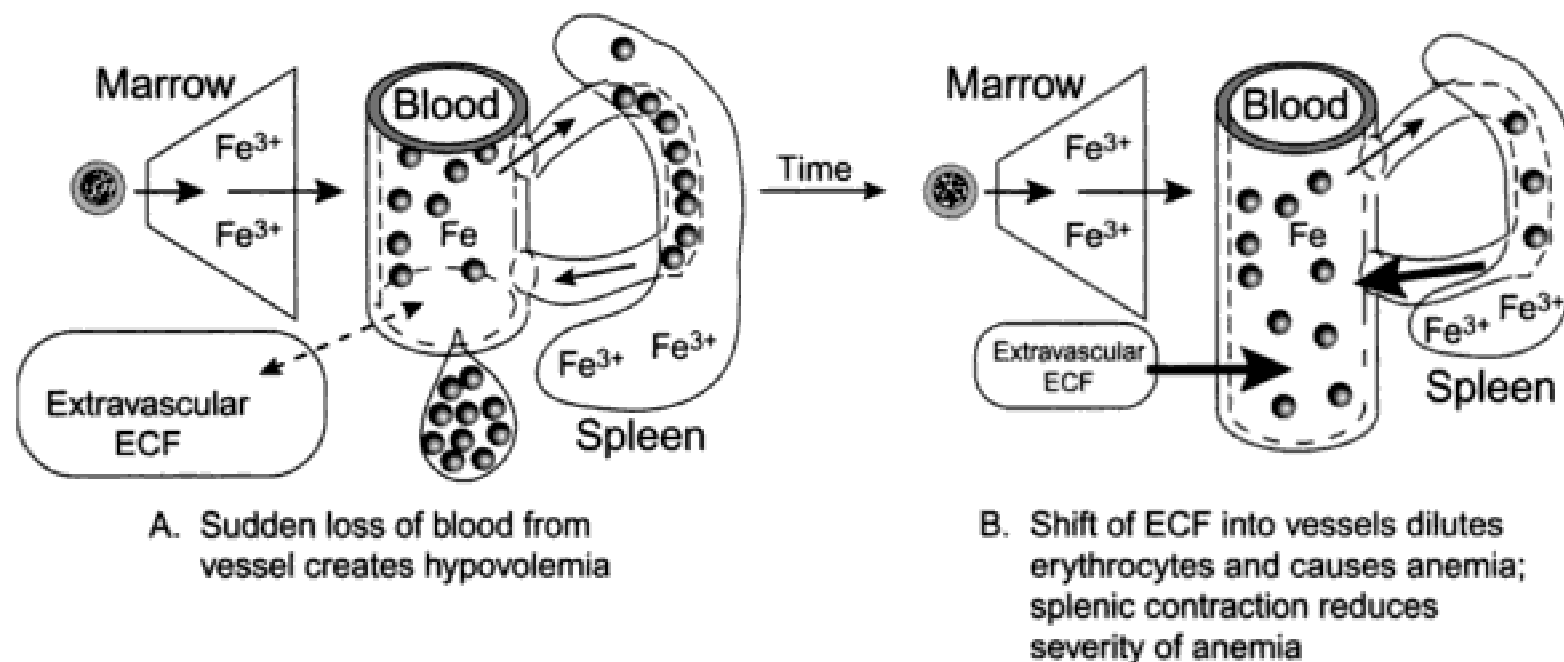

Fig. 4.7. Erythrocyte kinetics of acute blood loss.
A. Immediately after whole blood is lost, Hct and [TP] should not change because erythrocytes and plasma are lost proportionately. However, blood volume is decreased.
B. Hypovolemia stimulates thirst to replenish ECF volume and induces movement of ECF from extravascular space to intravascular space, thus expanding blood volume. The fluid shift dilutes erythrocytes (and plasma proteins) and thus anemia (and hypoproteinemia) develops. The degree of anemia depends on the quantity and duration of hemorrhage and the length of time since the onset of hemorrhage. Splenic contraction will diminish the severity of the anemia because splenic blood is rich in erythrocytes.

throcytes are absorbed (auto-transfusion) or erythrocytes are 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 to 4 days after blood loss (except in horses).

2. Clinical data that support a conclusion that an anemia is due to acute blood loss (major diagnostic features)
   a. Blood loss was observed (historical or physical examination).
      (1) Gross external hemorrhage seen; if GI hemorrhage, feces are tarry (melena) or feces are occult blood positive (heme present); if urinary tract hemorrhage, erythrocytes in urine sediment or heme-positive reaction
      (2) Hemothorax or hemoperitoneum
   b. Regenerative anemia if sufficient time for response
   c. Hypoproteinemia with a proportionate decrease in albumin and globulin concentrations

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 GI or urinary tracts) over several weeks to months. Anemia results from a combination of factors but is primarily the result of Fe deficiency (Fig. 4.8).
   2. When Fe deficiency develops, reticulocytosis is usually present but less than expected for the degree of anemia (marrow is poorly responsive). Developing erythrocytes may become RNA-depleted during the prolonged maturation and thus are not recognized as reticulocytes.
   3. Clinical data that support a conclusion that an anemia is due to chronic external blood loss

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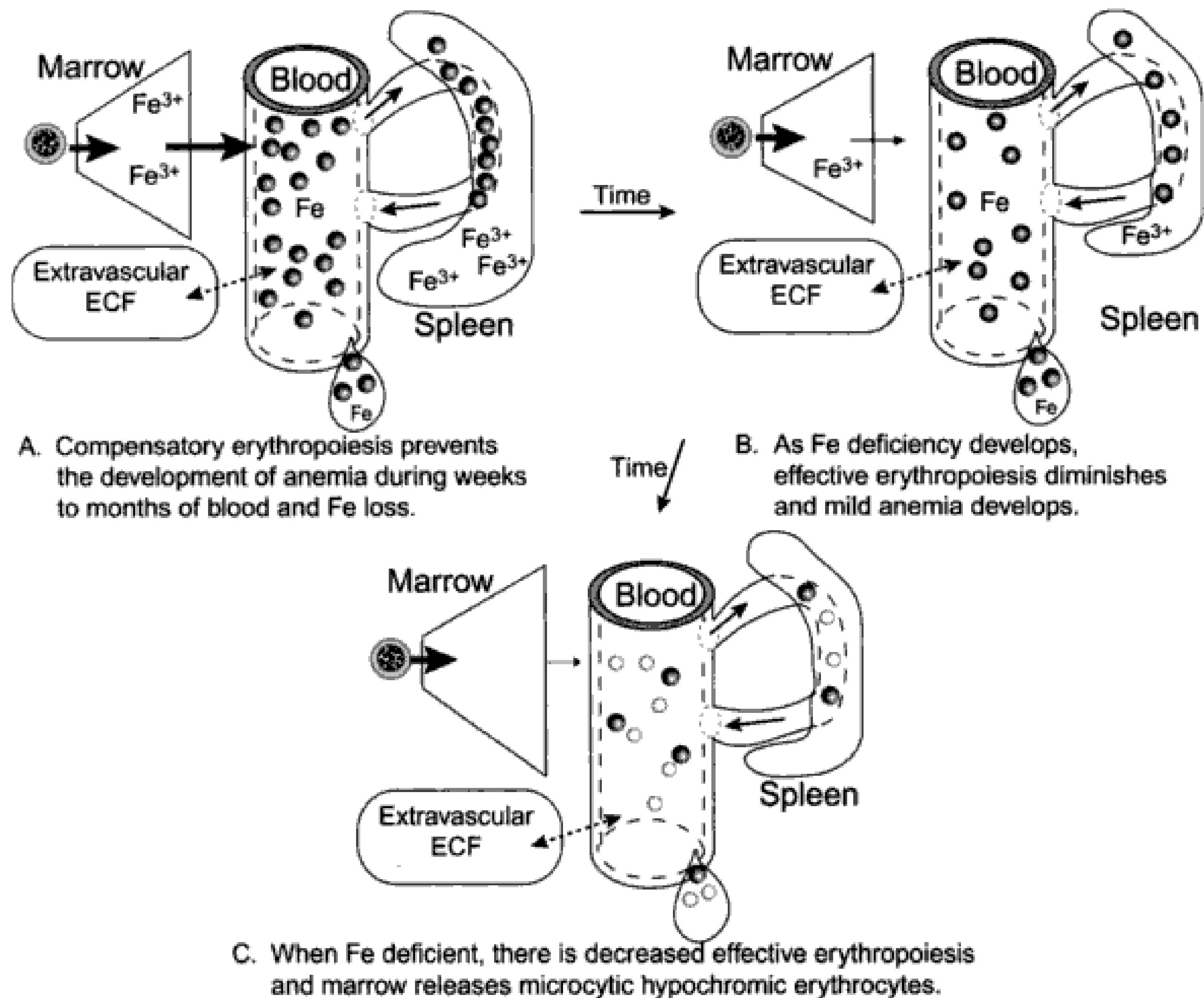

Fig. 4.8. Erythrocyte kinetics of chronic blood loss that results in iron deficiency.
A. Initially, there is a continual loss of small quantities of blood over weeks to months. Anemia does not develop as long as compensatory increased erythropoiesis (using stored Fe) replaces lost erythrocytes.
B. After prolonged blood loss, Fe deficiency develops (decreased total body Fe). When Fe deficiency is severe enough, erythropoiesis decreases sufficiently so that there is not adequate compensation for blood loss and thus anemia ensues. Fe deficiency affects many organs and is present before anemia occurs.
C. Microcytosis and hypochromasia result from the defective heme synthesis caused by Fe deficiency. Hypochromasia develops because there is inadequate Fe available for incorporation into heme for Hgb formation. While attempting to reach "ideal" cytoplasmic [Hgb], 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] and then hypochromic cells are formed (microcytic hypochromic anemia).

a. May find tarry feces (melena); urine or feces may be heme positive; frank hemorrhage frequently not observed; if due to parasitism, may find intestinal nematode ova, fleas, or other parasites
b. Poorly regenerative, microcytic normochromic to microcytic hypochromic anemia; erythroid hyperplasia in marrow but ineffective erythropoiesis because of the maturation defects
c. Mild to moderate hypoproteinemia
d. Hypoferremia, decreased total body Fe (depleted storage sites), and decreased serum ferritin concentration

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e. History of frequent blood donations
4. Young animals are more prone to develop Fe deficiency anemias than mature animals because they have relatively little Fe storage, they consume less Fe while on a milk diet, and they have large Fe requirements during growth.

HEMOLYTIC ANEMIAS

I. Concepts and classifications
  A. Hemolysis (erythrolysis) is erythrocyte necrosis and occurs at the end of every erythrocyte's life. When the rate of *in vivo* hemolysis increases, then it is a pathologic state. Pathologic hemolysis may be defined as an increased rate of erythrocyte destruction resulting in a decreased erythrocyte life span.
  B. Extravascular versus intravascular hemolysis
    1. Major differences
      a. Intravascular hemolysis
        (1) Erythrocyte destruction occurs in the blood within blood vessels or heart.
        (2) It is clinically recognized when it causes hemoglobinemia and hemoglobinuria (or if measured, decreased serum haptoglobin concentration).
      b. Extravascular hemolysis
        (1) Erythrocyte destruction occurs outside of arterial-capillary-venous system. It has been called intracellular hemolysis because destruction occurs in macrophages near venular sinuses of spleen, liver, and bone marrow. Splenic macrophages have greatest contact with erythrocytes in the red pulp. Macrophages also have the ability to attach to erythrocytes within blood by reaching through noncontinuous capillary walls, and then binding, engulfing, and lysing the erythrocyte.
        (2) It does not cause hemoglobinemia or hemoglobinuria.
    2. Why differentiate intravascular hemolysis from extravascular hemolysis?
      a. Establishing a major site of erythrocyte destruction may be a diagnostic clue; i.e., certain diseases typically cause extravascular hemolysis, others typically cause intravascular hemolysis (Table 4.8).
      b. Differentiation may be helpful in determining prognosis and treatment. Intravascular hemolysis usually occurs with life-threatening diseases and thus its presence suggests a poorer prognosis and immediate treatment and management of the case are indicated.
    3. Problems with classification system
      a. "Diseases" don't read the book; i.e., a disorder may be described as causing extravascular hemolysis but your case may be the uncommon exception with intravascular hemolysis that was not mentioned.
      b. Diseases may cause anemia by both intravascular and extravascular hemolysis.
      c. Disorders may switch from one to another (i.e., mild extravascular hemolysis becomes an intravascular crisis).
    4. Major features of the hemolytic disorders are listed in Table 4.9.
  C. Thorough examination of erythrocytes 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 definite clues of a hemolytic process.
    1. Organisms: *Haemobartonella, Eperythrozoon, Anaplasma, Babesia, Cytauxzoon, Theileria*

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Table 4.8. Hemolytic disorders and conditions

- Immune hemolytic disorders
  - »Idiopathic[a] (includes autoimmune)
  - Drug-induced[a]
  - Alloimmune
    - Neonatal isoerythrolysis
    - Blood transfusion reactions[a]
  - Hemolysis induced by bacterial and viral infections
    - »*Haemobartonella* spp.
    - *Eperythrozoon* spp.
    - »*Anaplasma* spp.
    - *Leptospira* spp.[b]
    - EIAV[a,b]
    - FeLV[b]
    - Hemolysis associated with other infections (e.g., ehrlichial)[b]
- Erythrocytic metabolic defects (acquired or inherited)
  - Oxidative damage
    - Heinz body hemolysis[a]
    - Eccentrocytic hemolysis (acquired or inherited)[a]
  - Defects in ATP generation
    - PK deficiency
    - PFK deficiency[a]
    - Hypophosphatemic hemolysis[a]
  - Defects in heme synthesis that result in porphyria
    - Bovine congenital erythropoietic porphyria
    - Feline erythropoietic porphyria
- Erythrocyte fragmentation in blood creating schizocytes, keratocytes, or acanthocytes
  - »Intravascular coagulation (localized or disseminated)
  - »Vasculitis
  - Hemangiosarcoma
  - Rheologic disorders
    - Caval syndrome of dirofilariasis
    - Cardiac valvular disease
- Erythrocyte membrane damage due to phospholipase activity from *Clostridium* spp.
  - Bacillary hemoglobinuria (*Clostridium haemolyticum* or *C. novyii*)[a]
  - Yellow lamb disease (*Clostridium perfringens*, type A)
- Hemolytic anemia of other or unknown pathogeneses
  - Protozoal infections
    - *Cytauxzoon felis*[b]
    - *Babesia* spp.[a]
    - *Theileria* spp.
    - *Trypanosoma* spp.
  - Heparin-induced hemolysis
  - Iatrogenic hypoosmolar hemolysis[a]
  - Envenomation (snakes, spiders, insects)
  - Histiocytic neoplasia[b]
  - Idiopathic nonspherocytic hemolytic disorders with increased osmotic fragility
    - Hereditary nonspherocytic hemolytic anemia of beagles
    - Idiopathic hemolytic anemia of Abyssinian and Somali cats

[a] Hemoglobinuria or hemoglobinemia may be present because of marked intravascular hemolysis.
[b] Other mechanisms may also cause anemia in these infections.

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Table 4.9. Major features of intravascular and extravascular hemolytic disorders

Feature	Clinical intravascular hemolysis[a]	Predominantly extravascular hemolysis[b]
Site of hemolysis	Within blood vessels or heart	Macrophages near blood sinuses of spleen, liver, or marrow
Degree of RBC damage directly caused by the hemolytic agent/process	Marked	Mild to moderate
Severity of anemia	Marked or rapidly falling	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[c]	Usually at presentation; usually Bu > Bc
Bilirubinuria	No[c]	Usually at presentation

[a] Clinical intravascular hemolysis is recognized by finding pathologic hemoglobinemia and pathologic hemoglobinuria. In most of these disorders, there will be concurrent extravascular hemolysis.

[b] During these disorders, intravascular hemolysis may be occurring but it is not severe enough to cause hemoglobinemia or hemoglobinuria.

[c] If concurrent extravascular hemolysis of sufficient duration and severity, hyperbilirubinemia and bilirubinuria can be present.

2. Clues of a hemolytic process: spherocytes, Heinz bodies, eccentrocytes, pyknocytes, schizocytes, keratocytes, acanthocytes

D. Hemolytic icterus (jaundice)

1. Pathologic hemolysis leads to increased Hgb degradation, thus increased bilirubin formation, and perhaps development of icterus. Icterus may develop in animals with either intravascular or extravascular hemolytic disorders. In both forms, there is increased Hgb degradation but the sites of erythrocyte destruction differ. Icterus that develops in animals with intravascular hemolysis may be due to the concurrent extravascular hemolysis.
2. Hyperbilirubinemia occurs when Bu travels through the blood from tissue macrophages to the liver. If Bu formation exceeds an animal's ability to excrete it into the bile as Bc, hyperbilirubinemia will develop. If the capacity of the liver for Bu uptake, conjugation, and excretion (rate-limiting step) are not exceeded, serum bilirubin concentration may remain WRI even though pathologic hemolysis is present.
3. The "back-up" step (or rate-limiting step) in bilirubin excretion is the transport of Bc to the biliary system. Once the transport maximum is reached, Bc is "regurgitated" out of hepatocytes and into plasma.
4. Bu and Bc compete for the same receptors on hepatocytes; thus, once the excretion system becomes saturated, both forms increase in plasma. Generally, with icterus of hemolytic origin, [Bu] > [Bc]. In longer-standing hemolytic disorders (week or more), the [Bc] may equal or exceed [Bu]; especially if there is liver damage (caused by hypoxia or other insults).

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E. Bilirubinuria (bilirubin in urine)
   1. Bc is H_2O soluble and is not protein bound, thus it easily passes through the glomerular filtration barrier and is not resorbed (low renal threshold).
   2. Bu is H_2O insoluble and is bound with albumin in plasma, thus very little Bu gets through the glomerular filtration barrier in most animals (high renal threshold).
   3. Dogs have a very low renal threshold for bilirubin and thus some bilirubin is present in urine of healthy dogs. The bilirubin may be Bc because it freely passes through the glomerular filtration barrier. However, many healthy dogs also have a mild albuminuria, and thus the detected bilirubin may be Bu bound to albumin.
   4. Bilirubinuria usually occurs before clinical hyperbilirubinemia (icterus) because Bc is excreted in urine as soon as its concentration in blood starts to increase and clinical icterus is usually not recognized until serum bilirubin concentrations exceed 1.5 to 2.0 mg/dL.

F. Urobilinogenuria
   1. Renal urobilinogen excretion increases in hemolytic anemias but urobilinogenuria is not recognized frequently, perhaps because urobilinogen is unstable in urine.
   2. Increased urinary urobilinogen excretion occurs because all the pathways associated with the excretion of heme degradation products are enhanced.

G. Hemolytic hemoglobinemia and hemoglobinuria (Fig. 4.9)
   1. Hgb nephropathy
      a. Hgb nephropathy is a pathologic state seen in hemolytic disorders characterized by proximal tubular degeneration leading to renal insufficiency.
      b. Hgb casts may be present in renal tubules and/or urine.
   2. A key to recognizing intravascular hemolysis is differentiating the pathologic causes of red, brown, or black urine, i.e., hemoglobinuria, hematuria, and myoglobinuria. These pigmenturias can typically be classified by criteria shown in Table 4.10. Other causes of pigmenturia are described in Chapter 8.

II. Hemolytic disorders and diseases (Tables 4.8 and 4.11)

A. Immune hemolytic anemias
   1. Concepts
      a. Immune hemolytic anemias occur when an animal's immune system produces antibodies that bind directly or indirectly to its own erythrocytes (ESAIg) and lead to erythrocyte destruction. The process may be initiated by a defective immune system, defective erythrocytes, or adsorbed antigens from drugs, infectious agents, or neoplasms. Factors that initiate the process are usually not known.
      b. Hemolysis may occur through three major processes (Fig. 4.10).
      c. Clinical evidence may suggest the presence of intravascular hemolysis, extravascular hemolysis, or both. ESAIg molecules of immune hemolysis may be IgG, IgM, or IgA.[44,45] Extravascular hemolysis occurs when erythrocytes coated with immunoglobulins are engulfed by macrophages. If the immunoglobulins fix complement, the membrane attack complex (C5b-9) may form and cause intravascular hemolysis. Variations in antibody involvement create variations in clinical manifestations.
      d. Direct antiglobulin tests (or Coombs' test) may be used to detect the presence of ESAIg or complement on a patient's erythrocytes (see Chap. 2).
      e. To understand some of the clinical manifestations and laboratory findings of immune hemolytic anemias, the major features of warm and cold antibodies need to be understood.

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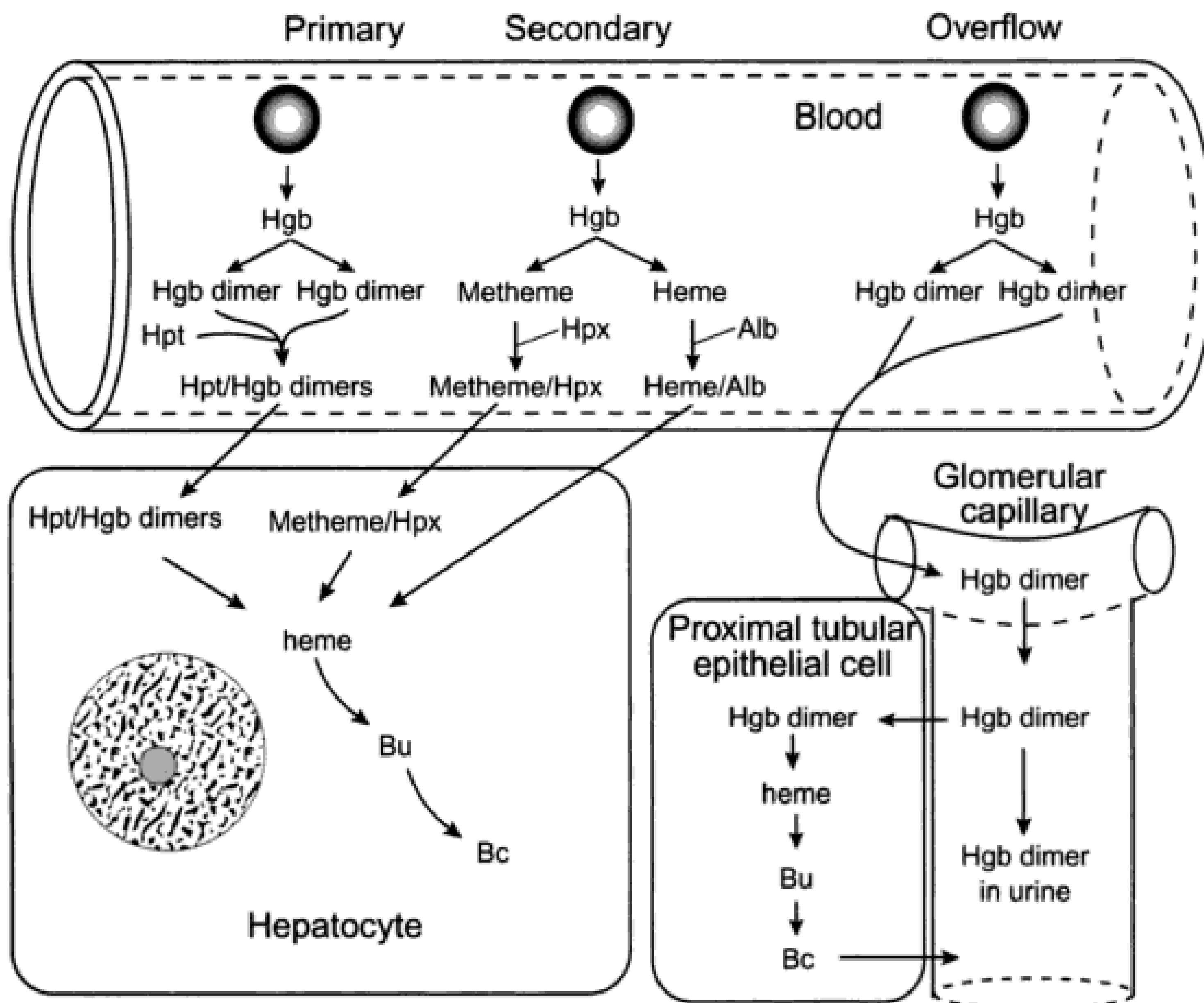

Fig. 4.9. Pathogenesis of hemoglobinemia and hemoglobinuria during intravascular hemolysis (Hpt = haptoglobin, Hpx = hemopexin).

- Normal physiologic processes that conserve Fe released during intravascular hemolysis can be divided into primary and secondary systems. These systems are not saturated during health and thus hemoglobinemia is not seen in health. In pathologic states, saturation of the primary and secondary systems leads to hemoglobinemia and hemoglobinuria (loss of Hgb and Fe).
- Primary system of Fe conservation (does most of work unless saturated or overwhelmed)
  - Intravascular erythrocyte damage or death causes release of Hgb to plasma. The unstable Hgb tetramer splits into dimers (Hgb-D) and immediately binds to Hpt (if available). The Hpt/Hgb dimer complexes are cleared from plasma primarily by hepatocytes, which degrade Hgb-D to Bu, Fe^{3+}, and amino acids.[130,131] There is evidence that macrophages have a receptor for the Hpt/Hgb complex and thus they may also be involved in clearing the complexes from plasma.[132]
  - Plasma [Hgb] or [Hgb/Hpt] may be high enough to cause the plasma to be pink, and thus hemoglobinemia is recognized. Plasma will appear pink when plasma [Hgb] $\geq$ 50 mg/dL.
- Secondary system of Fe conservation (becomes more important after plasma [Hpt] decreases)
  - Continued or more severe intravascular erythrocyte damage or death causes release of more Hgb to plasma. The plasma Hgb may oxidize to form methemoglobin, which dissociates and releases metheme and globin. Metheme binds to Hpx to form a metheme-Hpx complex and heme binds with albumin (Alb) to form a heme-albumin complex.[133]
  - Metheme-Hpx complexes enter hepatocytes, where they are degraded to Bu, amino acids, and Fe^{3+}. Heme from the heme-albumin complex may directly enter hepatocytes.[133]
- Overflow (when Fe conservation systems are saturated; results in marked hemoglobinemia and concurrent hemoglobinuria)
  - If the rate of hemolysis exceeds the ability of Hgb-binding proteins to conserve Fe, free Hgb-D accumulate in plasma (hemoglobinemia); the transport maximum of Hpt for Hgb-D is near 150 mg/dL.
  - Hgb-D pass through the glomerular filtration barrier and are excreted in urine (hemoglobinuria). Proximal tubular epithelial cells have minimal ability to resorb Hgb-D and degrade them to Bu for conjugation and urinary excretion.

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Table 4.10. 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
Urine color	Pink to red[c]	Pink to red[c]	Pink to red[c]
Urine heme reaction	Positive	Positive	Positive
RBCs in urine sediment	Yes[d]	No	No
Information to support muscle damage[e]	No	No	Yes

[a] Unless there is 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.

[d] Erythrocytes may lyse after they enter urine (especially in dilute urine) and thus may not be seen in a urine sediment examination.

[e] Information may include historical or physical evidence of muscle damage (e.g., stiffness, trauma) or increased serum creatine kinase activity.

Table 4.11. Recognized hemolytic anemias of dogs, cats, horses, and cattle

Type of anemia	Dogs	Cats	Horses	Cattle
Immune-mediated (not infectious)	IIHA Drug-induced NI Transfusion	IIHA NI Transfusion	IIHA Drug-induced NI Transfusion	IIHA
Infectious	*Haemobartonella* *Babesia* *Ehrlichia*	*Haemobartonella* *Cytauxzoon* FeLV[a] *Ehrlichia*[a]	*Babesia* *Leptospira*[a] EIA *Ehrlichia*[a]	*Eperythrozoon* *Anaplasma* *Babesia* *Theileria* *Leptospira* *Clostridium* *Trypanosoma*
Metabolic	Heinz body Eccentrocytic PK deficiency PFK deficiency Hypophosphatemia	Heinz body PK deficiency Erythropoietic porphyria	Heinz body Eccentrocytic	Heinz body Hypophosphatemia Congenital erythropoietic porphyria
Traumatic	Angiopathy	Angiopathy	Angiopathy	—
Other	Envenomation Histiocytic neoplasia Hereditary nonspherocytic	Idiopathic (↑ fragility)	Heparin-induced	Hypoosmolar

[a] Firm evidence of hemolytic anemia not found.

(1) Warm antibodies (usually IgG)
 (a) Maximal binding activity at 37°–39°C
 (b) Warm antibodies are more common than cold antibodies. They may bind submaximally to erythrocytes at room temperatures and thus can cause autoagglutination.

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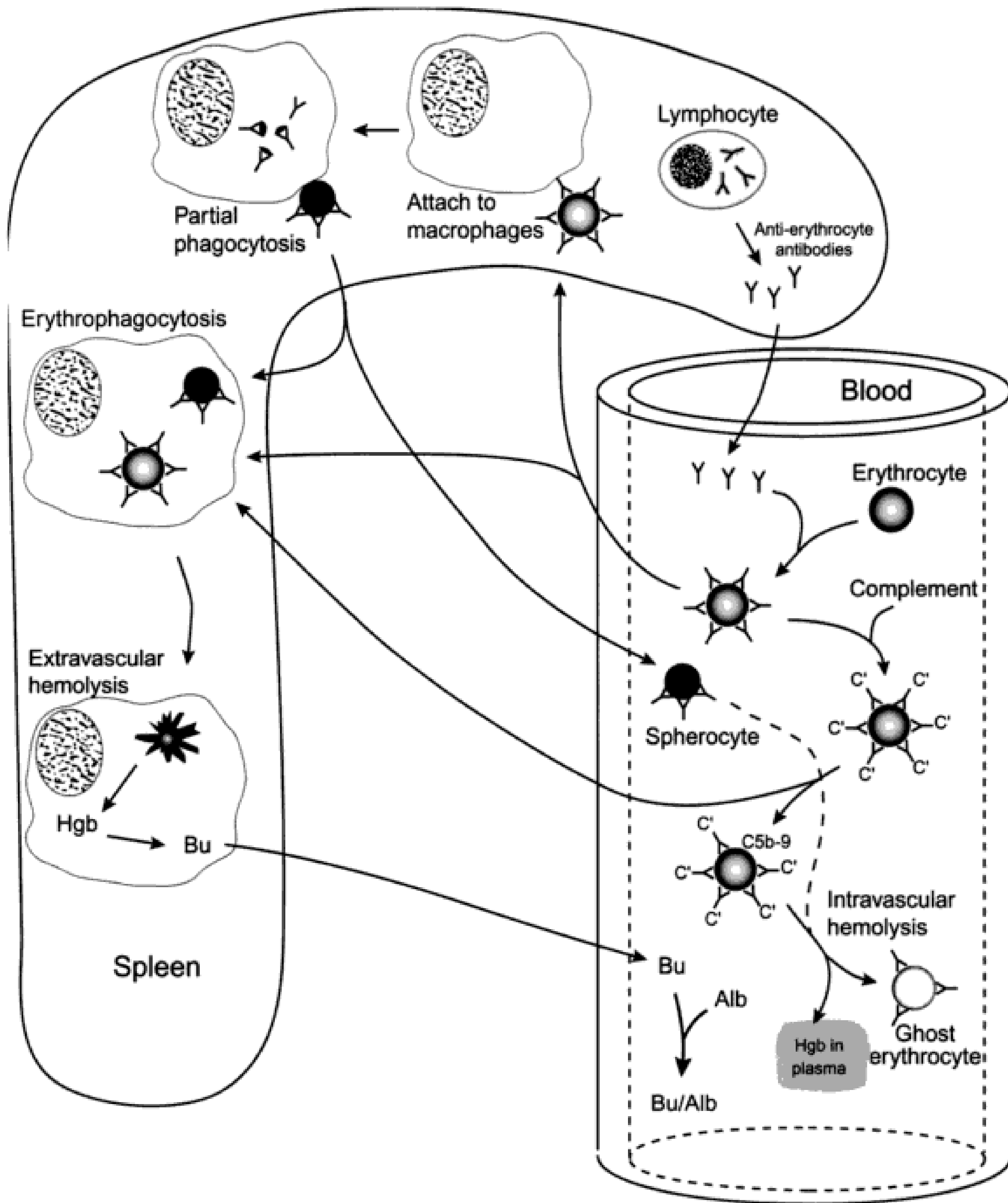

Fig. 4.10. Pathogenesis of immune hemolysis.

- Erythrocytes coated with ESAIg and/or C3 undergo extravascular hemolysis in macrophages.
- Erythrocytes coated with ESAIg and/or C3 are converted to spherocytes by macrophages removing erythrocyte membrane. Spherocytes undergo either extravascular or intravascular hemolysis because of their rigidity and fragility, respectively.
- Some ESAIg may bind complement (especially IgM), which activates the complement cascade and leads to intravascular hemolysis via C5b-9, the membrane attack complex.

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(2) Cold antibodies (usually IgM)
(a) Maximal binding activity at 4°–20°C
(b) Cold antibodies may be able to bind *in vivo* while passing through cool extremities, and thus they can activate complement and initiate immune hemolysis. They typically will cause autoagglutination as erythrocytes cool during or after collection.

2. Disorders
   a. IIHA (also called IMHA, immune-mediated hemolytic anemia)
      (1) IIHA is the most common hemolytic anemia of dogs. It is also found in cats, horses, and cattle.
      (2) IIHA is commonly referred to as AIHA although the factors that initiated the immune hemolysis are not known. Typically, it is assumed that the hemolysis is an autoimmune process because known causes of immune hemolysis are not found.
      (3) Classic laboratory features of IIHA: regenerative anemia (mild to severe), icterus, possibly hemoglobinuria, spherocytosis (usually recognized only on dog blood films), positive Coombs' test, inflammatory leukogram including neutrophilia with regenerative left shift and monocytosis, absence of evidence of other immune hemolytic anemias
      (4) Exceptions to classic features
         (a) Anemia may be nonregenerative if it is less than 2–3 days duration, if antibodies are also attacking marrow erythrocyte precursors (e.g., pure red cell aplasia), or if another disease process is interfering with erythropoiesis.
         (b) Evidence of extravascular hemolysis (icterus, bilirubinuria, etc.) may not be present if the rate of erythrocyte destruction is only slightly increased or if accelerated hemolysis is of short duration.
         (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) Coombs' test may be negative due to a low density of ESAIg or technical problems, including prozone reaction, poor reagents, or poor sample.
      (5) Other laboratory or diagnostic features of IIHA cases: erythrocyte autoagglutination, ghost erythrocytes, frequently thrombocytopenia, splenomegaly
   b. Drug-induced immune hemolytic anemias
      (1) Drugs are thought to induce immune hemolysis through four processes.[46]
         (a) Drug binds to erythrocyte and becomes haptenic; antibodies bind to the hapten.
         (b) Drug binds to a plasma protein and the conjugate becomes antigenic; antibodies bind to the drug/protein conjugate to form an immune-complex that binds to erythrocytes.
         (c) Drug binds to erythrocyte to form drug/membrane complexes that are antigenic.
         (d) Drug induces the formation of autoantibodies that bind to natural antigenic structures on the erythrocyte membrane.
      (2) Drugs reported to initiate an IHA in domestic mammals include penicillin in horses,[47,48] propylthiouracil in cats,[49] and cephalosporins in dogs.[50] Many drugs are reported to cause IHA in people.[46]
   c. NI (hemolytic disease of the newborn)

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(1) In NI (neonatal isoerythrolysis), ingested maternal colostral alloantibodies are absorbed by the intestine, enter blood, and then attach to the neonate's paternal-derived erythrocyte surface antigens. The antibody-coated erythrocytes are then lysed by macrophages or complement.

(2) In cats, NI occurs when a queen with Type B blood passes her anti-A alloantibodies to her Type A or Type AB kittens via colostrum. Type B erythrocytes are uncommon in American domestic shorthair cats (< 5%); highest incidence is found in British shorthair (41%), Devon rex (40%), and Cornish rex (34%) cats.[51] 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.[52]

(3) 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 or transfusions, and her foal is positive for the Aa or Qa alloantigen (paternally derived). Other acquired alloantibodies are typically too weak to cause isoerythrolysis.[53] In one study involving 799 mares, anti-erythrocyte 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.[54]

(4) In dogs, NI may occur if a DEA 1.1-negative bitch develops anti-DEA 1.1 antibodies after being transfused with DEA 1.1-positive blood. Dogs are not known to have clinically significant naturally occurring alloantibodies and other acquired alloantibodies probably do not cause hemolysis.[52]

d. IHA secondary to incompatible blood transfusions

(1) Transfused donor's erythrocytes are attacked by the recipient's (patient's) circulating anti-erythrocyte antibodies. Transfused erythrocytes are recognized as foreign because the donor and recipient have different antigens on their erythrocyte membranes.

(2) The alloantibodies that cause hemolysis are the same as those that cause NI. In dogs and horses, the alloantibodies are acquired antibodies that develop during pregnancy, parturition, or after whole blood transfusions. In cats, the antibodies are natural alloantibodies.

(3) To reduce the possibility of a hemolytic transfusion reaction, two laboratory tests may be used.

(a) Blood typing

(i) An animal's blood type is determined by immunologic assays that detect alloantigens on potential donor cells. For example, a cat with B-alloantigen is a Type B cat.

(ii) Blood from dogs positive for DEA 1.1 should not be given to DEA 1.1-negative dogs. Blood from horses positive for Aa- or Qa-alloantigens should not be given to horses negative for Aa- or Qa-alloantigens, respectively. Blood from Type B cats should not be given to Type A cats.

(b) Cross-matches

(i) Major cross-match

- The procedure involves mixing and incubating potential donor's erythrocytes with the recipient's serum. Antibody binding is detected by agglutination, hemolysis, or a Coombs' test.

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- A compatible major cross-match indicates that alloantibodies against the donor's erythrocytes were not detected. An incompatible major cross-match indicates that alloantibodies against the donor's erythrocytes were detected.
- There is not a standard cross-matching procedure in veterinary medicine and thus results may vary. An incompatible cross-match may occur when the detected alloantibody is not clinically significant. Conversely, some significant alloantibodies are not detected by agglutination and require complement-reactions or Coombs' testing to detect.

(ii) Minor cross-match (not done as frequently as the major)
- The procedure involves mixing and incubating recipient's erythrocytes with potential donor's serum.
- An incompatible minor cross-match is usually not a clinical concern because it is not expected to cause a major transfusion reaction.

e. IHA induced by bacterial and viral infections

(1) *Haemobartonella* spp. (may be reclassified as *Mycoplasma* spp.)

(a) *H. felis* in cats; causes feline infectious anemia

(i) Organisms vary in pathogenicity; most strains are opportunistic while others are considered strict pathogens.

(ii) Parasitemia is usually present during hemolysis, may suddenly disappear (within hours) from peripheral blood, and may fall off erythrocytes *in vitro* and thus films made from fresh blood need to be examined.

(b) *H. canis* in dogs

(i) Parasitemia is usually seen in splenectomized and in immunologically compromised dogs.

(ii) Like *H. felis, H. canis* may detach from erythrocytes as the sample ages.

(c) Pathogenesis of the hemolysis is considered immune-mediated. Antibodies bind to the parasitized erythrocytes either because of bound parasite antigens or antigens exposed on altered membranes.

(d) Major laboratory findings: *Haemobartonella* spp. on erythrocytes in films of fresh blood; moderate to severe anemia; reticulocytosis and polychromasia; mild to moderate hyperbilirubinemia and bilirubinuria; direct Coombs' test positive; spherocytosis (dog); autoagglutination; PCR-positive for *Haemobartonella* spp.

(2) *Eperythrozoon* spp.

(a) *E. suis* and *E. parvum* in pigs, *E. wenyonni* in cattle, and *E. ovis* in sheep; an eperythrozoal organism has been found in llamas.

(b) Parasites are most numerous when Hct is falling.

(c) Like *Haemobartonella*, organisms may detach from erythrocytes *in vitro*. Best chance to detect and identify organisms is on blood films made immediately after blood collection.

(d) Pathogenesis of hemolysis is considered immune-mediated (same as hemobartonellosis).

(e) Laboratory findings for *E. suis* infection: *E. suis* on erythrocytes; moderate to severe anemia; reticulocytosis or polychromasia; mild to moderate

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hyperbilirubinemia and bilirubinuria; PCR-positive for *Eperythrozoon* spp.

(f) Like hemobartonellosis, eperythrozoal infections may not cause a clinical hemolytic anemia unless the animal is immunologically compromised.

(3) *Anaplasma* spp.

(a) *A. marginale* (cattle), *A. ovis* (sheep and goats), *A. centrale* (cattle of South America, Africa, Middle East)

(b) *A. marginale* are most numerous when Hct is falling; 4–5 days later, it is difficult to find organisms. The marginal body may appear to protrude from the erythrocyte membrane, but it is not external.

(c) Pathogenesis of anemia is mostly immune-mediated. Antibodies bind to erythrocytes damaged by the anaplasmal infection and cause extravascular hemolysis.

(d) Major laboratory findings: organisms found early in disease; moderate to severe anemia; reticulocytosis, polychromasia, and basophilic stippling; mild to marked hyperbilirubinemia and bilirubinuria; PCR-positive for *Anaplasma* spp.

(4) *Leptospira* spp.

(a) *L. interrogans* serovars *pomona* and *icterohemorrhagica* do not infect erythrocytes but infection of other tissues (kidney, liver) can lead to a hemolytic state.

(b) *Leptospira* spp. infect most domestic animals and many wild animals[55] but the hemolytic state is seen primarily in calves, lambs, and pigs.[56,57] 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[58,59] or leptospiral phospholipase activity.[60-62]

(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; positive PCR test for *Leptospira* spp.

(5) 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 (swamp fever, equine malarial fever, mountain fever, slow fever).

(b) Persistent replication of EIAV in macrophages and the horse's response to the infection create the pathologic states that cause clinical disease.[63,64] The clinical disease may be an acute hemolytic state or a chronic debilitating disease caused by the recurrent episodes.

(c) Anemia pathogenesis: both decreased erythrocyte production and hemolysis

(i) Decreased production due to tumor necrosis factor and other cytokines that inhibit erythroid precursors

(ii) Hemolysis is due to immune-complexes or complement fragments adhered to erythrocytes, which are then destroyed by macrophages.

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(d) Major laboratory findings
(i) Anemia may appear as an acute intravascular hemolytic anemia in acute stages; more commonly it is a chronic extravascular hemolytic anemia or a chronic normocytic normochromic anemia.
(ii) Other findings: hemoglobinemia (acute); perhaps anisocytosis and macrocytosis; perhaps spherocytosis; thrombocytopenia; neutropenia or neutrophilia; Coombs' test positive; Coggins' test positive
(6) FeLV
(a) Most anemias associated with FeLV infections are due to decreased erythrocyte production, but some might be due to hemolysis.[65,66]
(b) A FeLV infection may predispose a cat to certain infections (e.g., *Haemobartonella*) or be associated with immune hemolytic processes.

B. Erythrocytic metabolic defects (acquired or inherited)
1. Oxidative damage
a. Heinz body hemolytic anemia
(1) Heinz bodies are foci of denatured hemoglobin and their formation appears to involve a sequence of events in erythrocytes.[67]
(a) Erythrocytes are exposed to an oxidant that overwhelms the reductive pathways that keep Hgb in a reduced state (Fig. 4.5).
(b) Hemoglobin is converted to Hgb-Fe^{3+}.
(c) Hgb-Fe^{3+} undergoes spontaneous conformational changes to form hemichromes or form a heme-depleted Hgb.
(d) Hemichromes or heme-depleted Hgb molecules precipitate and aggregate to form Heinz bodies. Oxidation of sulfhydryl groups and formation of disulfide bond occurs during the precipitation process. The Heinz bodies are frequently associated with the erythrocyte membrane through hydrophobic bonds.
(2) Many oxidants or substances contain oxidants reported to cause Heinz body formation in domestic mammals.
(a) Dogs: acetaminophen, benzocaine, hydrogen peroxide, onions (n-propyl disulfide), Vit K_1 (phytonadione), Vit K_3 (menadione), naphthalene, phenylhydrazine, possibly zinc[68]
(b) Cats: acetaminophen, benzocaine, methionine, methylene blue (in old urinary antiseptics), onions, phenazopyridine, propylene glycol
(c) Horses: onions, phenothiazine, red maple (*Acer rubrum*) leaves (wilted or dry)
(d) Ruminants: *Brassica* spp. (kale, rape), copper toxicosis, hydrogen peroxide (intravenous), molybdenum deficiency, onions, rye grass
(3) Pathogenesis of the anemia may involve multiple mechanisms[67]
(a) Erythrocytes 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 erythrocyte membrane leads to fragile cells that may lyse within vessels or sinusoids.
(c) Binding of hemichromes to band-3 proteins of the erythrocyte membrane result in a redistribution of band-3 proteins and formation of an antigen that is recognized by autologous antibodies. After antibody binding, the defective erythrocyte is removed by splenic or hepatic macrophages.

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(4) Major laboratory features: mild to severe anemia; polychromasia and reticulocytosis; Heinz bodies in peripheral blood (NMB or other vital stain may help confirm presence); eccentrocytosis; hyperbilirubinemia and bilirubinuria; in acute severe cases, hemoglobinemia and hemoglobinuria; methemoglobinemia may be present (dark or chocolate-colored blood)

(5) Feline Heinz bodies

(a) Clinically healthy, nonanemic cats and cats with nonhemolytic anemias frequently have circulating erythrocytes which contain Heinz bodies that are of little to no clinical significance; typically, a minority of the erythrocytes contain Heinz bodies.

(b) Feline Heinz bodies were described as erythrocyte refractile bodies (ER bodies) by Schalm. In unstained, direct, wet preparations, Heinz bodies were refractile (do not confuse with refractile membranes on stained smears that are artifacts).

(c) Theories that attempt to explain why healthy cats may have circulating Heinz bodies

(i) Feline erythrocytes containing Heinz bodies are probably not removed from circulation as rapidly as in other species because the cat's spleen has a closed circulation. Therefore, erythrocytes do not flow through the red pulp in which "pitting" of Heinz bodies is thought to occur in other species.

(ii) Because feline Hgb has more sulfhydryl groups than other species, it may be prone to form more disulfide bridges, and thus more denatured Hgb.

(iii) Feline erythrocytes may have less reductive capacity.

(d) Cats that eat semi-moist food containing propylene glycol have more Heinz bodies and their erythrocytes have shorter life spans, but clinically significant anemia is not expected.

(e) Cats with a variety of disorders (e.g., diabetes mellitus, hyperthyroidism, lymphosarcoma) may have increased percentages of erythrocytes containing Heinz bodies.

(f) 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 erythrocytes. Known exposure to an oxidant is very helpful.

b. Eccentrocytic hemolytic anemias (acquired or inherited)

(1) Pathogenesis of the anemia probably involves multiple mechanisms

(a) Eccentrocytes are more rigid and thus less able to pass through splenic sinusoids, thus they are trapped and removed by macrophages.

(b) Eccentrocytes may be more fragile because of the damaged membrane or cytoskeleton and thus may spontaneously rupture in blood.

(2) Acquired states

(a) Oxidative damage to erythrocytes may cause formation of eccentrocytes, Heinz bodies, or both.

(b) Factors that determine the outcome of oxidative damage are not understood. Oxidants reported to cause Heinz bodies in one animal may cause eccentrocytosis or methemoglobinemia in another.

(3) G6PD deficiency

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(a) X-linked defect that is common in people and was found in one American saddlebred colt.[18] The nonsense mutation occurred in the colt's dam[69] and is not a breed problem because none of the colt's male siblings lived to produce progeny.

(b) Pathogenesis of anemia: G6PD is the rate-controlling enzyme for the HMP shunt from which NADPH is produced (Fig. 4.5). NADPH is a potent reducing agent that keeps GSH, and indirectly other substances, in a reduced state. With reduced G6PD activity, erythrocytes are not able to repair damage caused by spontaneous oxidation, thus, the cells are prone to eccentrocyte and pyknocyte formation and reduced life spans.

(c) Major laboratory findings in the horse: persistent macrocytic normochromic anemia (Hct near 20%); eccentrocytosis and pyknocytosis; macrocytosis; persistent icterus (primarily increased [Bu])

(d) Confirmatory diagnostic findings: greatly reduced erythrocyte G6PD activity; decreased concentrations of GSH and NADPH; increased methemoglobin concentration

(4) FAD deficiency

(a) A Spanish mustang mare had a defective biochemical pathway in her erythrocytes that created a FAD deficiency.[70] The FAD deficiency led to deficient activity of Cb_5R and GR, enzymes that contain FAD (Fig.4.5). Hereditary aspects of the defect were not determined.

(b) Major laboratory findings in the horse: eccentrocytosis, pyknocytosis, Hgb crystals, and methemoglobinemia

2. Defects in ATP generation

a. PK deficiency

(1) PK deficiency is a hereditary disorder of people, several breeds of dogs (basenji, beagle, cairn terrier, West Highland white terrier, American Eskimo) and in Abyssinian cats.[71,72] 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.

(2) Pathogenesis of anemia: Normal erythrocytes have a PK isoenzyme (R-type) that normally catalyzes the last ATP generation step of anaerobic glycolysis (Fig. 4.5). Without PK activity, erythrocytes become ATP-deficient, erythrocyte membranes become defective, and hemolysis occurs. In this disorder, young erythrocytes have a little PK (R-type) but the PK (R-type) content decreases as the cell ages and erythrocytes have a greatly decreased life span.

(3) Major laboratory findings: moderate anemia, moderate to extreme reticulocytosis (RP = 40%–60%), mild to moderate icterus; spheroechinocytes are reported to occur but are uncommon in canine cases; pancytopenia may develop in later stages if myelofibrosis develops.

(4) Confirmatory diagnostic findings: decreased erythrocyte PK (R-type) activity; total erythrocyte PK activity may be increased because of increased M2-PK (heat-labile). PCR testing can confirm some PK deficiencies.[73]

b. PFK deficiency

(1) First recognized in English springer spaniel dogs with erythrocyte PFK activity near 10% of healthy dogs.[74,75] Other cells in these dogs (e.g., muscle fibers) were also deficient in PFK. More recently recognized in American cocker spaniels[76] and mixed breed dogs (which may have had spaniel parentage).

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(2) Pathogenesis of anemia: PFK is a rate-controlling enzyme of glycolysis (Fig. 4.5). In healthy dogs, decreased [H^+] in blood (increased pH) causes increased PFK activity and thus increases ATP and 2,3-DPG formation by the glycolytic pathway. Hyperventilation (due to excitement or exercise) causes decreased blood P_aCO_2 and alkalemia. In PFK-deficient patients, erythrocytes do not have PFK to respond to the alkalemia. Thus, glycolysis is defective and less ATP is produced. This may result in unstable erythrocyte membranes and hemolysis.

(3) Major laboratory findings: anemia, hemoglobinemia, and hemoglobinuria after hyperventilation; hemolytic icterus; when not in active hemolytic state, dogs have mild regenerative or compensated anemias.

(4) Confirmatory findings: PFK-deficient erythrocytes, decreased erythrocyte 2,3-DPG and increased chloride concentrations; PCR testing can confirm PFK deficiencies.

c. Hypophosphatemic hemolysis

(1) Postparturient hemoglobinuria in cattle

(a) Occurs 3–8 weeks after calving

(b) Pathogenesis of anemia: Defective mobilization of phosphorus from bone and increased phosphorus loss via milk causes a pronounced hypophosphatemia. Without plasma phosphorus, there is defective ATP production by erythrocytes that results in ATP deficiency, unstable erythrocyte membranes, and hemolysis.

(c) Major laboratory findings: hypophosphatemia, hemoglobinemia, hemoglobinuria, moderate to marked anemia

(2) There are sporadic reports in horses, dogs, and cats[77] of concurrent hypophosphatemia and severe hemolysis. Severe hypophosphatemia may result in decreased erythrocyte ATP production and thus unstable erythrocyte membranes and hemolysis. In dogs, low [2,3-DPG] may also play a role by making cells more susceptible to alkaline-associated hemolysis.[1]

(3) Bilirubin interference can cause artifactual hypophosphatemia with some assay methods, so one must be careful about interpreting the concurrent presence of hypophosphatemia and hemolytic anemia.

(4) Hyperinsulinism (primary or secondary to hyperglycemia) may cause hypophosphatemia by promoting movement of glucose and phosphorus into cells other than erythrocytes (glucose transport into erythrocytes is not insulin dependent). Persistent hypophosphatemia may lead to depletion of erythrocyte phosphorus, decreased ATP production, and hemolysis.

3. Defects in heme synthesis that result in porphyria

a. Porphyrias are a group of hereditary and acquired disorders in which porphyrins accumulate in cells and body fluids because of a deficient enzyme activity in the heme synthetic pathway (Fig. 4.2). Collectively, porphobilinogen through protoporphyrin IX are called porphyrins. Some authors define porphyria as an inherited disorder and porphyrinuria as an acquired disorder.[78]

(1) Some forms of porphyria produce a hemolytic anemia, but other forms do not. The accumulation of certain porphyrins in erythrocytes results in a reduced erythrocyte life span and thus a hemolytic anemia. The mechanism of erythrocyte destruction is not firmly established but may relate to porphyrin-induced damage of membrane lipids or photolysis when erythrocytes

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are near the skin surface.[79,80] 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.

(2) Porphyrias can be classified as either erythropoietic or hepatic depending on the major expression of the enzymatic defect.[79,80] The inherited hepatic porphyrias have not been documented in domestic mammals.

b. Congenital erythropoietic porphyria
   (1) Bovine congenital erythropoietic porphyria
      (a) The homozygous calves with this autosomal recessive disorder have reddish brown discoloration of teeth and bones, photosensitivity, and anemia of varying severity. The disorder is found primarily in Holsteins, but also in Shorthorn and Jamaican cattle.[79]
      (b) The porphyria results from a hereditary deficiency of uroporphyrinogen III cosynthase, an enzyme that catalyzes one of the first porphyrin reactions of Fig. 4.2.
   (2) Feline erythropoietic porphyria
      (a) Erythropoietic porphyria was diagnosed in a female Siamese cat and two male offspring. The cats had photosensitivity, hemolytic anemia, and renal disease.[81]
      (b) The specific enzymatic defect was not established in the cats.

c. Other hereditary erythropoietic porphyrias have been diagnosed in cattle, pigs, and cats; however, anemia was not reported as a feature of these forms.[79,82,83] Lead poisoning creates an acquired porphyria because lead inhibits enzymes in the heme synthetic pathway. Anemia may be present in plumbism, but is not considered a hemolytic anemia caused by the porphyrin accumulation.

C. Erythrocyte fragmentation in blood creating schizocytes, keratocytes, or acanthocytes
   1. Erythrocyte damage is thought to be due to trauma caused by relatively rigid structures (fibrin) or by rheologic forces (see Table 4.8 for disorders).
   2. Pathogeneses of anemia: Because the erythrocyte trauma is a consequence of other pathologic states, processes that cause the anemia may be multifaceted.
      a. Erythrocyte trauma either directly causes lysis or creates poikilocytes that have a shortened life span. Acanthocytes may form in circulation because of membrane lipid changes rather than mechanical or rheologic forces, but these forces may contribute to the accelerated fragmentation of acanthocytes ("budding fragmentation").
      b. Primary diseases are frequently infectious and noninfectious inflammatory disorders and thus the inflammatory state may be producing anemia (see Nonregenerative Anemias, II.A).
   3. Major laboratory findings: mild to moderate anemia with no to moderate reticulocytosis and polychromasia; schizocytosis, keratocytosis, or acanthocytosis; thrombocytopenia; may have other evidence of a consumptive coagulopathy

D. Erythrocyte membrane damage due to phospholipase activity from *Clostridium* spp.
   1. *C. haemolyticum* and *C. novyii* type D
      a. Disease: bacillary hemoglobinuria in cattle and sheep (red water disease, Nevada red water)
      b. *C. haemolyticum* and *C. novyii* type D are normal inhabitants of soil and may remain dormant in cattle until anaerobic conditions promote their growth. In the

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United States, bacillary hemoglobinuria is seen primarily in poorly draining areas of Gulf Coast and Western states, and there is a reported association with liver flukes.

c. Pathogenesis of anemia: Erythrocyte damage is caused primarily by a β toxin (produced by either *C. haemolyticum* or *C. novyii* type D) that has phospholipase C or lecithinase activity. The β toxin thus degrades membrane lecithin and causes lysis of erythrocytes. Other relatively minor hemolysins (lipase, proteinase, another lecithinase) have also been reported as products of *C. haemolyticum*.[84]

d. Major laboratory findings: acute severe anemia, hemoglobinemia, and hemoglobinuria

e. Confirmatory diagnostic findings: post mortem lesions; Gram-positive bacilli in liver, spleen, blood, and abdominal fluid; *C. haemolyticum* cultured from liver; phospholipase C activity (β toxin) detected in tissues

2. *C. perfringens* type A (*C. welchii*)

   a. Disease: yellow lamb disease in calves and lambs (enterotoxemic jaundice, yellows)

   b. *C. perfringens* type A is part of normal intestinal flora in animals, 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 spring nursing lambs of northern California and Oregon.[85] It also occurs in lambs and calves of Canada, Australia, New Zealand, and South Africa.

   c. Pathogenesis of anemia: An α toxin that has phospholipase C activity and is produced by *C. perfringens* type A causes hydrolysis of membrane phospholipids and thus lysis of erythrocytes, leukocytes, platelets, endothelial cells, and myocytes.[86]

   d. Major laboratory findings

      (1) Acute severe cases: anemia, hemoglobinemia, hemoglobinuria, icterus

      (2) Less severe forms: anemia, polychromasia, basophilic stippling, rubricytosis, leukocytosis

E. Hemolytic disorders of other or unknown pathogeneses

1. Protozoal infections

   a. *Cytauxzoon felis*

      (1) The pathogenesis of the anemia is not well understood. In most cases, the chronic parasitic infection that damages the marrow, spleen, and liver contributes to a progressive nonregenerative anemia.

      (2) Major laboratory findings: *C. felis* in erythrocytes; mild to occasionally severe anemia, typically nonregenerative; moderate to marked hyperbilirubinemia (may be primarily Bc because of hepatic damage and cholestasis) and bilirubinuria; thrombocytopenia; rarely, large macrophages laden with schizonts are present in the blood film; other hematologic and clinical chemistry abnormalities due to hepatic, splenic, lymph node, and marrow damage associated with the schizont stage of the parasitic infection

   b. *Babesia* spp.

      (1) Species, host (expected number of piroplasms per erythrocyte): *B. bigemina,* cattle (1 or 2); *B. caballi,* horse (2 or 4), *B. equi,* horse (1 or 2); *B. canis,* dog (1, 2, 4 or 8), *B. gibsoni,* dog (1 or 2); there is another small canine *Babesia* that has not been named.[87]

      (2) Sizes: *B.equi, B. gibsoni,* and other small *Babesia* spp. < 2 µm; others 2–4 µm in length

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(3) Parasitemia: difficult to find organisms in chronic forms of disease; more frequently found in capillary blood ear sticks, toenail clips, or in buffy coat preparations because specimen contains a higher percentage of large erythrocytes

(4) Pathogenesis of hemolysis is not well understood but may involve proteases produced by the invading parasite, an immune reaction to parasitized cells, or oxidative damage to erythrocytes.

(5) Major laboratory findings

(a) Chronic form: few to rare organisms in blood; mild anemia; mild lymphocytosis (due to chronic antigenic stimulus); seropositive to *Babesia* spp.

(b) In acute or subacute forms: many piroplasms in blood; moderate to severe anemia; reticulocytosis, polychromasia, macrocytosis; hyperbilirubinemia, bilirubinuria and possibly hemoglobinuria

c. *Theileria* spp.

(1) Erythrocyte piroplasms are intracellular and highly pleomorphic depending on species and stage of parasitemia; many forms resemble *Cytauxzoon* but others resemble small babesial piroplasms.

(2) In the United States (Missouri, Texas, North Carolina), bovine cases have been caused by *T. buffeli*.[88,89] Numerous piroplasms were present in clinically ill cows; piroplasms were rare in subclinical cases. Pathogenesis of the anemia is not established and may include both decreased erythrocyte production and decreased erythrocyte life span (either immunologic or oxidative damage). Major laboratory findings: piroplasms in erythrocytes; acanthocytosis and spherocytosis in one cow; macrocytosis, polychromasia, and basophilic stippling; lymphocytosis; hyperbilirubinemia and bilirubinuria.

(3) In other countries, many *Theileria* spp. are recognized as causing hemolytic anemias in cattle and other ungulates. Erythrocytic piroplasms are the major pathogenic forms in *T. mutans*, *T. orientalis*, and *T. sergenti*. The major pathogenic stage of *T. parva* is in the intralymphocytic schizont, whereas erythrocytic and lymphocytic forms are considered important for *T. annulata*.[90,91]

d. *Trypanosoma* spp. (see Plate 3.*W*)

(1) *Trypanosoma* spp. are flagellated protozoa that occur as free-living trypomastigotes in blood and as amastigotes in pseudocysts or macrophages in other tissues. The pathogenicity of the parasitic species varies and there is minimal host specificity.[92]

(2) In the United States, *T. theileri* (previously called *T. americanum*) is found in cattle and typically is not considered a pathogen. There are reports of finding it in Kansas, Wyoming, Oklahoma, Louisiana, Missouri, Illinois, Pennsylvania, and New York. It is also found in other countries.

(3) *T. cruzi* infects dogs and cats in South and Central America and in southern United States of America. Trypomastigotes are found in blood in the acute stages but most lesions are in nonhemic tissues (heart, brain, and lymph node) and hemolytic anemia is not a feature of the disorder.[93] *T. cruzi* causes Chagas disease in people.

(4) Major African trypanosomes of veterinary significance are *T. congolense*, *T. vivax*, *T. brucei*, and *T. simiae*. A subspecies of *T. brucei* causes African sleeping sickness in people.

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(a) In the acute stages of *T. vivax* infections in cattle, the animals may have an acute hemolytic anemia, leukopenia, and thrombocytopenia. Phagocytosis of hemic precursors and platelets is a prominent finding in marrow and thus ineffective hematopoiesis contributes to the pancytopenia.[94]

(b) *T. congolense* infections in cattle and sheep have resulted in the coating of erythrocytes and leukocytes with trypanosomal antigen-antibody complexes.[95,96]

2. Heparin-induced hemolysis
   a. Heparin anticoagulant therapy in some horses can cause erythrocyte agglutination, mild to moderate anemia, and increased biliary bilirubin excretion. Pathogenesis of the decreased Hct value is not established.
      (1) *In vivo* erythrocyte agglutination appears to cause trapping and destruction of erythrocytes.[97]
      (2) When determined by Coulter cell counters, *in vitro* erythrocyte agglutination can cause erroneously low [RBC] and erroneously high MCV values because a group of agglutinated cells is considered one large cell (particle). Therefore, the falsely decreased measured [RBC] can produce a falsely low calculated Hct. The effects of heparin can be inhibited and reversed *in vitro* by trypsin.[98]
      (3) When analyzed by the Cell-Dyn, the group of agglutinated cells is also recognized as one large cell during impedance counting. However, if the volumes of the "large cells" are outside the expected distribution curve, they are excluded from the data used for MCV determination. Thus, the derived MCV may be nearly correct but the [RBC] will be falsely decreased because many cells were within the "large cells." Accordingly, the calculated Hct will be falsely decreased and the MCHC and MCH will be falsely increased.
   b. Major laboratory findings: In experimental studies, spun Hct (microhematocrit) decreased from near 30% to near 20% within 6–8 hr after heparin treatment and then returned to baseline values by 4–5 days. Bilirubin concentrations increased from near 0.6 mg/dL to 1.4 mg/dL within 24 hr of heparin treatment.[97]
3. Hypoosmolar hemolysis
   a. Rapid infusion of hypotonic fluid intravenously (such as sterile H_2O) or the ingestion of large quantities of water by calves can cause rapid intravascular hemolysis.
   b. Pathogenesis of the anemia: Infusion of a hypotonic solution or absorption of large quantities of water creates hypoosmolar plasma. Rapid movement of H_2O into erythrocytes via osmosis causes erythrocyte swelling and lysis.
   c. Major laboratory findings: Severity of anemia, hemoglobinemia, and hemoglobinuria depend on the severity of the hypoosmolar state.
4. Envenomation
   a. Venoms from some animals (e.g., snakes, spiders, insects) cause hemolysis. Mechanisms include complement activation with subsequent destruction (e.g., cobra venom factor) and direct hemolysis from hemolysins (including phospholipase A2 in rattlesnake venom).[14]
   b. Spherocytic hemolytic anemias may occur after bee stings. The hemolysis may be due to hemolysins present in the venom: phospholipase A2 and melittin. The spherocytosis may be related to altered membrane structure or antibody-mediated changes.[20,21]

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5. Histiocytic neoplasia: When anemia accompanies malignant histiocytosis, extravascular hemolysis by neoplastic cells is one contributing factor to an animal's anemia.
6. Idiopathic nonspherocytic hemolytic disorders with increased osmotic fragility
   a. Hereditary nonspherocytic hemolytic anemia in beagles
      (1) Affected beagles had mild chronic anemia (Hct values of 30%–39%, persistent reticulocytosis, splenomegaly, erythroid hyperplasia in marrow samples, shortened erythrocyte life spans, and increased osmotic fragility.[99,100]
      (2) Studies suggest that the defect is an autosomal recessive trait, but the specific defect has not been established.
   b. Idiopathic hemolytic anemia of Abyssinian and Somali cats[101]
      (1) 18 cats (13 Abyssinian and 5 Somali, male and female) had macrocytic, regenerative anemias (Hct values of 5%–25%), splenomegaly, and increased erythrocyte osmotic fragility.
      (2) Studies suggest that the defect is an autosomal recessive trait, but the specific defect has not been established.

ERYTHROCYTOSIS AND POLYCYTHEMIA

I. Terms and concepts
   A. *Erythrocytosis:* increased [RBC] in peripheral blood; detected by finding an increased Hct, increased [RBC], or increased blood [Hgb]
   B. *Hemoconcentration:* increased concentration of blood components (including erythrocytes) because of decreased plasma volume
   C. *Polycythemia*
      1. *Polycythemia vera* (*polycythemia rubra vera*) is a clonal myeloproliferative disorder characterized by neoplastic proliferation of all marrow cell precursors causing erythrocytosis, leukocytosis, and thrombocytosis. In this context, polycythemia means many (*poly-*) cells (*cyth-*) in blood (*-emia*).
      2. In common use, polycythemia may refer to an erythrocytosis or increased total erythrocyte mass (i.e., an increased number of cells in the erythron due to either erythroid hyperplasia or erythroid neoplasia). However, there may or may not be a leukocytosis or thrombocytosis. Such use leads to confusion and misunderstandings. In this context, there are two types of polycythemia.
         a. Relative polycythemia: Erythrocytosis occurs because of hemoconcentration or splenic contraction; also called pseudopolycythemia or spurious polycythemia to emphasize that the state is not due to increased erythrocyte mass.
         b. Absolute or true polycythemia: Erythrocytosis occurs because of increased erythrocyte mass and there is concurrent erythrocytosis; polycythemia vera is one form of this type but also includes secondary erythrocytotic disorders.
      3. In this textbook, *erythrocytosis* and *polycythemia* will not be considered synonyms. Erythrocytosis is an increased [RBC] in blood, just as a leukocytosis is an increased [WBC] in blood. Polycythemia will be used only in the context of the neoplastic state of polycythemia vera.
   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, and seizures.

II. Erythrocytotic disorders and conditions (Table 4.12) (Fig. 4.11)
   A. Hemoconcentration

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Table 4.12. Disorders and conditions that cause erythrocytosis

Hemoconcentration
  *Dehydration
  Endotoxic shock
*Splenic contraction
Secondary appropriate erythrocytotic disorders
  Right to left shunts, congenital or acquired
  Chronic pulmonary disease
  Hyperthyroidism
Secondary inappropriate erythrocytotic disorders
  Renal neoplasms, cysts, or diseases
  Other neoplasms (hepatoma)
Primary erythrocytotic disorders
  Primary erythrocytosis
  Polycythemia vera

Note: Animals that live at higher altitudes or perhaps have been physically trained (e.g., racing animals) have increased need for hemoglobin to transport O_2 to tissues and thus produce more erythrocytes. In addition, certain breeds of dogs (greyhound, Afghan hound, saluki, and whippet) and horses (thoroughbred, standardbred, and quarter horse) have greater erythrocyte concentrations than other breeds of dogs and horses.

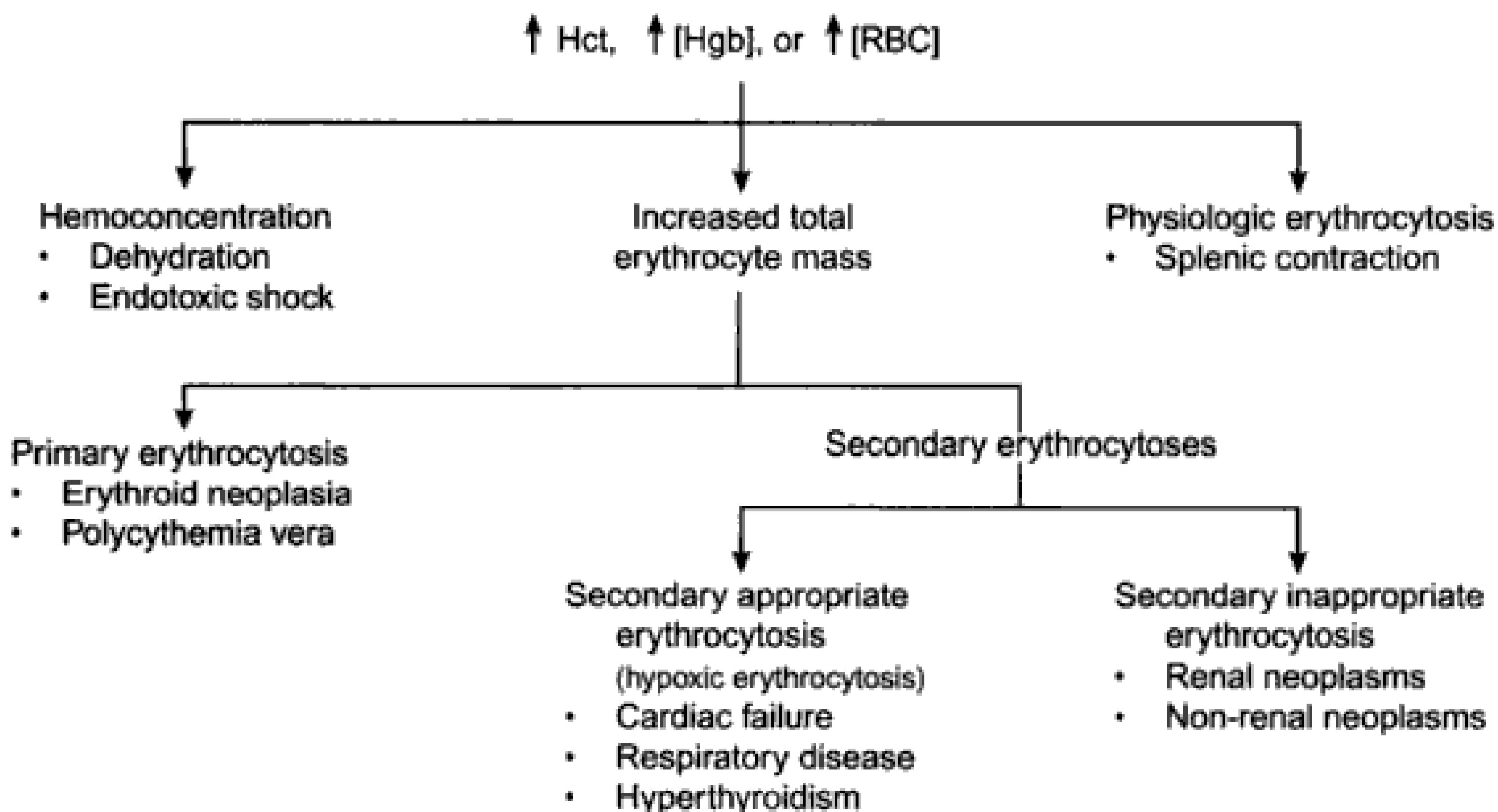

Fig. 4.11. Erythrocytosis categories.
After erythrocytosis has been detected or confirmed, the animal is examined for evidence of the most common causes: hemoconcentration or splenic contraction. If not found, then diagnostic plans are formulated to pursue identification of secondary or primary erythrocytotic disorders.

1. Dehydration
    a. Most common cause of erythrocytosis in mammals; occurs as a mild to moderate erythrocytosis of no direct pathologic significance
    b. Erythrocytosis is present but it is not due to an increased number of erythroid cells in the body.

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c. Pathogenesis of the erythrocytosis of hemoconcentration (Fig. 4.12)
d. Laboratory findings that support a conclusion that erythrocytosis is due to dehydration
(1) Hyperproteinemia; may be a concurrent hyperalbuminemia
(2) Hypernatremia and hyperchloremia if it is a hypertonic dehydration (loss of hypotonic fluid)
2. Endotoxic shock
a. Some animals with endotoxemia may become hemoconcentrated because of the shift of H_2O from intravascular to extravascular space.
b. Pathogenesis of the erythrocytosis: Endotoxins damage endothelial cells so that blood vessels become more permeable to H_2O. The increased permeability allows ECF water to shift from intravascular space to extravascular space, thus causing a decrease in plasma volume and an erythrocytosis.
c. Laboratory findings that support endotoxic shock
(1) Mild to moderate erythrocytosis; no direct pathologic significance
(2) Inflammatory leukogram (either neutropenia or neutrophilia)
(3) Thrombocytopenia

B. Splenic contraction (Fig. 4.12)
1. This state is called physiologic erythrocytosis because it results from normal physiologic responses to excitement, fright, and exercise that cause the release of epinephrine from adrenal medulla.
2. This erythrocytosis is more common in dogs and horses because of the large number and high concentration of erythrocytes in equine and canine splenic blood. After epinephrine injections in cats, Hct values were about 25% higher than pre-injection Hct values.[102]
3. Erythrocytosis is not due to an increased number of erythroid cells in the body, but there is an increased erythrocyte number and concentration in the peripheral blood.
4. Laboratory findings
a. Mild to moderate erythrocytosis; no direct pathologic significance
b. Transient erythrocytosis; Hct returns to WRI after stimulus is removed.
c. May see a physiologic leukocytosis (mature neutrophilia and lymphocytosis).

C. Secondary appropriate erythrocytotic disorders (sustained hypoxic erythrocytosis) (Fig. 4.12)
1. This state is called *secondary* because erythroid cell proliferation is stimulated by Epo rather than being autonomous; it is called *appropriate* because the increased Epo is due to renal (usually systemic) hypoxia rather than being autonomous.
2. Erythrocytosis and erythroid hyperplasia are due to increased production stimulated by Epo.
3. Disorders that cause hypoxic erythrocytosis
a. Cardiac disease that leads to persistent poor perfusion of lungs (e.g., congenital right to left shunt)
b. Pulmonary disorders that cause persistent poor oxygenation of blood
c. Hyperthyroidism that results in increased metabolic rate and thus increased need for O_2 in tissues. In one study, about 45% of 131 hyperthyroid cats had an erythrocytosis (Hct values from 38% to 57%).[103]
d. In theory, a defective Hgb molecule that has decreased ability to transport or release O_2 could cause an erythrocytosis. Acquired disorders (such as cyanide poisoning, carbon monoxide poisoning, nitrate poisoning) are typically acute disor-

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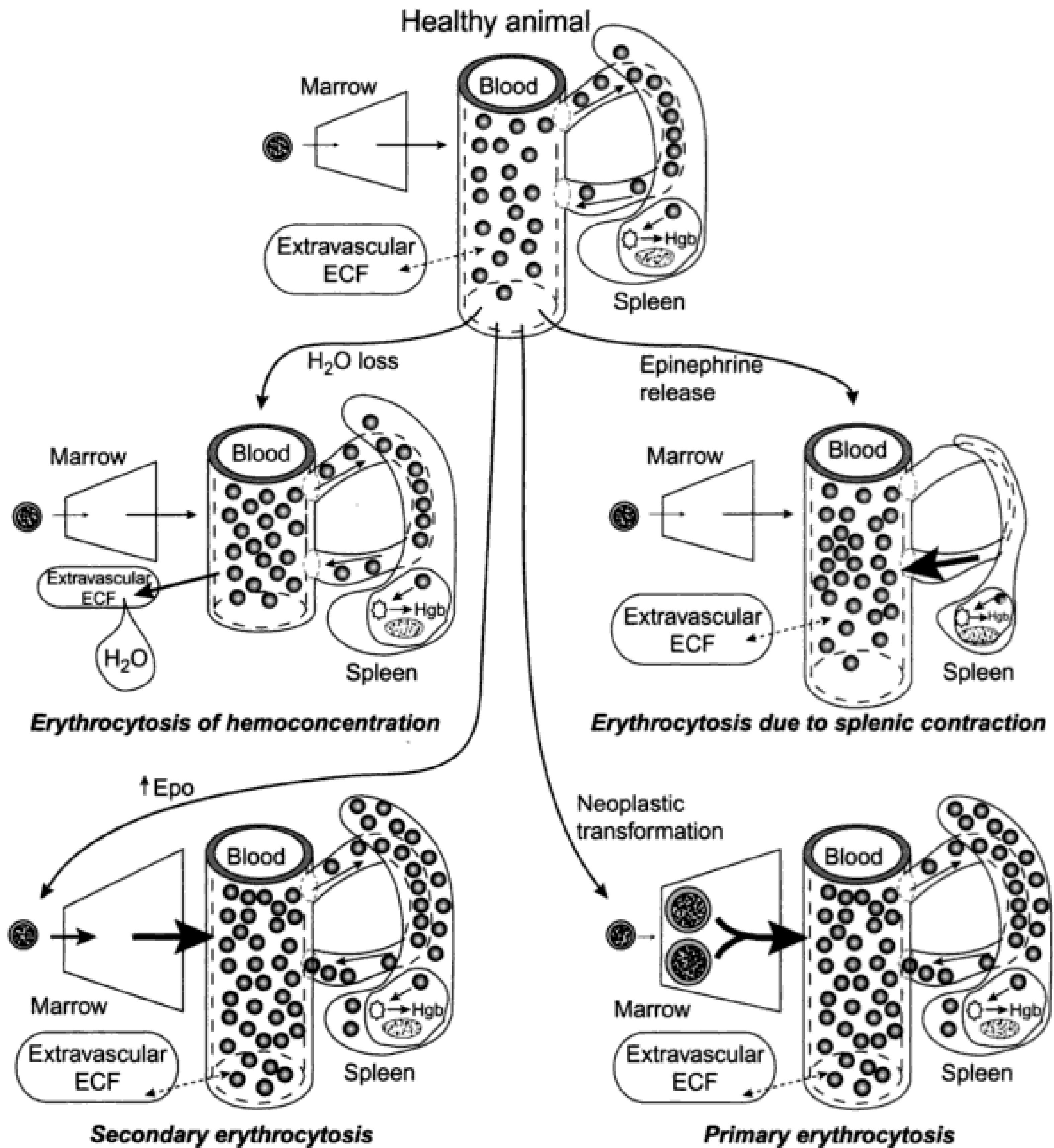

Fig. 4.12. Pathogeneses of the erythrocytoses.

- Erythrocytosis of hemoconcentration: Dehydration (decreased total body H_2O) results in a decreased ECF volume and thus a decreased plasma volume. With a decreased plasma volume but no change in number of blood erythrocytes, the erythrocyte concentration is increased. Loss of plasma H_2O because of increased vascular permeability in endotoxic shock also causes an erythrocytosis via hemoconcentration.
- Erythrocytosis due to splenic contraction: Fight-or-flight response or exercise causes the release of epinephrine. Epinephrine causes the contraction of splenic smooth muscle and thus the release of splenic blood to peripheral blood vessels. Splenic blood with a high Hct (70%–80%) is mixed with peripheral blood (Hct = 40%–50%) and results in erythrocytosis.
- Secondary erythrocytosis: Increased Epo activity stimulates erythropoiesis to cause erythroid hyperplasia and erythrocytosis. Increased Epo production may be appropriate (if stimulated by tissue [renal] hypoxia) or inappropriate (if not stimulated by tissue hypoxia). Several disease states can cause secondary erythrocytosis (see text).
- Primary erythrocytosis: A neoplastic proliferation of erythroid cells results in erythrocytosis. If there is a concurrent neoplastic proliferation of leukocytes and megakaryocytes, then polycythemia vera is present.

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ders and thus of insufficient duration to produce increased Hct. Hereditary disorders (hemoglobinopathies) are not documented in domestic animals.

4. Physiologic processes
   a. Mammals that move to high altitudes develop tissue hypoxia. This stimulates Epo production and erythropoiesis, resulting in erythroid hyperplasia and erythrocytosis that resolves the tissue hypoxia. Mammals that live at high altitudes have persistently higher Hct values than mammals that live at sea level, but without tissue hypoxia or pathologic consequence. Such animals really do not have erythrocytoses; rather, reference intervals for high altitudes are greater than those for low altitudes.
   b. Prolonged exercise training in horses has produced differing results. In some studies, the trained horses had higher Hct values but in other studies, they did not. Increased oxygen demand during training might provide the stimulus for erythropoiesis, but other mechanisms may contribute.[104,105]
   c. Some breeds have greater erythrocyte concentrations than others.
      (1) Horses in thoroughbred, standardbred, and quarter horse breeds have greater Hct values than draft horse breeds.
      (2) Dogs in greyhound, Afghan hound, saluki, and whippet breeds have greater Hct values than dogs of other breeds. Their Hct values average in the high 50s to low 60s,[106] whereas most dog breeds have average Hct values in the 40s in health.
5. Pathogenesis: Hypoxemia or increased tissue O_2 consumption (hyperthyroidism) cause sustained tissue (renal) hypoxia which leads to increased Epo production, increased erythropoiesis via erythroid hyperplasia, and, with time, erythrocytosis.
6. Clinical data that would support secondary appropriate erythrocytosis
   a. Clinical history or evidence of a right to left shunt, chronic pulmonary disease, or hyperthyroidism
   b. Decreased P_aO_2 (strongly if < 70 mmHg[107] or < 60 mmHg[108])
   c. Hemoglobinopathy (rare)

D. Secondary inappropriate erythrocytotic disorders (Fig. 4.12)
   1. This state is called *secondary* because erythroid cell proliferation is stimulated by Epo rather than being autonomous; it is called *inappropriate* because the increased Epo production is autonomous rather than being due to systemic hypoxia.
   2. The state can result in mild to marked erythrocytosis and erythroid hyperplasia; marked erythrocytosis may cause sluggish blood that leads to poor tissue perfusion.
   3. Pathologic causes (both rare or rarely recognized)
      a. Inappropriate Epo production by renal tissue due to renal cysts, renal neoplasms, and rarely other renal disease
      b. Inappropriate Epo production by other benign or malignant neoplasms (e.g., hepatoma, hepatoblastoma)
   4. Clinical data that would support the conclusion that a persistent erythrocytosis is secondary and inappropriate
      a. Finding the known associated pathologic disorders
      b. Serum total protein concentration and P_aO_2 are WRI.

E. Primary erythrocytotic disorders (Fig. 4.12)
   1. This state is called "primary" because erythroid cell proliferation is autonomous rather than secondary to Epo; it results in mild to marked erythrocytosis that may result in poor tissue perfusion and therefore secondarily increased Epo production.

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2. Disorders
   a. Primary erythrocytosis: neoplasia of erythroid cell line resulting in an increased total number of mature erythrocytes in the body and blood. This disorder may be an early form of polycythemia vera.
   b. Polycythemia vera: neoplasia of erythroid, myeloid, and megakaryocytic cell lines
3. Information that would support these disorders
   a. Absence of other causes of erythrocytosis
   b. With polycythemia vera, bone marrow with too many erythroid cell, myeloid cell, and megakaryocyte precursors
   c. Theoretically, blood [Epo] would not be increased with primary erythrocytosis and it would be increased with secondary erythrocytosis; however, blood [Epo] may be increased because of hypoxia induced by primary erythrocytosis, and [Epo] may not be increased with secondary erythrocytosis if erythrocytosis has resolved tissue hypoxia such that here is little or no stimulus for increased Epo production at the time of testing.

OTHER ERYTHROCYTE DISORDERS

I. Cytochrome-b_5 reductase (Cb_5R) (NADH-methemoglobin reductase) deficiency[109-112]
   A. Cb_5R catalyzes the primary reaction involved in the conversion of Hgb-Fe^{3+} to Hgb; it uses FAD as a cofactor.
   B. Hereditary disorder in man; genetics not known for dog and cats (reported in purebred and mongrel dogs)
   C. Classic features
      1. Young and old dogs with clinical signs of hypoxia due to poor O_2-carrying capacity of methemoglobin
      2. Methemoglobinemia (dark to chocolate blood) and usually Heinz bodies present
      3. No anemia or mild anemia; may be a compensatory reticulocytosis

II. Hereditary stomatocytosis
   A. Hereditary disorder in Alaskan malamute dogs with short-limb dwarfism (specific erythrocyte defect not known, probably erythrocyte membrane defect); also reported in schnauzers and Drentse partijshond[22,23]
   B. Classic features
      1. Mild anemia with mild to moderate reticulocytosis
      2. Stomatocytosis (mild to moderate)
      3. Shortened erythrocyte life span

III. Hereditary elliptocytosis of dogs[113]
   A. Deficiency of an erythrocyte membrane protein (band 4.1) that is needed to stabilize the cytoskeleton of the erythrocyte membrane
   B. Classic features
      1. Elliptocytosis: many erythrocytes involved
      2. Slight anemia but with moderate reticulocytosis (largely compensated)
      3. In cases reported so far, almost an incidental finding

IV. Megaloblastic anemia
   A. A megaloblastic anemia has the concurrent findings of anemia and megaloblastic erythroid precursors in bone marrow or blood.

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B. Megaloblastic erythroid precursors form because of asynchronous maturation of nucleus and cytoplasm (see Plate 3.*X*). Nuclear maturation is arrested, so nuclei are large with exaggerated euchromatin regions that persist to late rubricyte stages; cytoplasmic maturation is relatively more complete, so Hgb is apparent in cells with relatively immature nuclei. The cells have relatively abundant cytoplasm and thus are larger than would be expected for the maturity of the nucleus.

C. Causes
   1. Exact defect is rarely documented. It may be the result of neoplastic transformation of erythrocyte precursors, defective nucleic acid metabolism caused by folate or vitamin B_{12} deficiency, or defective metabolism.
   2. In cats, there is a high clinical association with FeLV infections (especially subtype C).

D. Frequently, cats with this disorder have a nonregenerative, macrocytic normochromic anemia (mild to marked severity).
   1. Nonregenerative due to defective and reduced erythrocyte production
   2. Macrocytic because megaloblastic metarubricytes become macrocytes (diameters of individual erythrocytes may be 1.5 to 2 times those of healthy cats).
   3. Bone marrow findings in cats
      a. Erythroid cell numbers: variable, increased to decreased
      b. Erythroid cells: frequent megaloblastic rubricytes and/or megaloblastic metarubricytes
      c. Granulocytic cells: occasionally hypersegmented neutrophils

E. Classification of megaloblastic disorders in cats
      a. Erythroid dysplasia (MDS or MDS-Er): cat has macrocytic anemia and megaloblastic cells in marrow and/or blood; may have concurrent neoplasia of nonerythroid cells.
      b. Erythroid neoplasia: Cat has M6 or M6-Er.

LABORATORY METHODS TO ASSESS IRON (Fe) STATUS[2]

Evaluation of an animal's Fe status may be helpful to confirm an Fe-deficient or an Fe-overload state.

I. Serum [Fe]
   A. Analytical concepts
      1. Terms and units
         a. Because nearly all Fe in nonhemolyzed serum is bound to transferrin, serum [Fe] represents the amount of Fe bound to transferrin. Fe in ferritin may contribute significantly to serum [Fe] if there is marked hyperferritinemia.
         b. Unit conversion: µg/dL × 0.01791 µmol/µg × 10dL/L = µmol/L (SI unit, nearest 1 µmol/L)[114]
      2. Sample
         a. Serum is preferred.
         b. Serum [Fe] is relatively stable if sample is refrigerated, frozen, or kept at room temperatures for a few hours.
      3. Common principle of serum Fe assays: Fe^{3+} is liberated from transferrin in an acidic environment and then is 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.
   B. Hyperferremia (increased [Fe] in blood) (Table 4.13)
      1. Excess Fe intake
         a. Iatrogenic

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Table 4.13 Disorders and conditions that cause hyperferremia

Disorders and conditions
Fe overload due to excess intake
Iatrogenic: excess Fe injections or oral hematinics
Genetic defect in regulation of Fe absorption
Release of Fe from tissues
Hepatocyte damage
Hemolysis
Increased glucocorticoid hormones: iatrogenic or endogenous (horses, dogs)

Note: Can be falsely increased with hyperferritinemia or *in vitro* hemolysis (see text).

(1) Excess ingestion of a hematinic in calves[115] and ingestion of a digestive inoculate (Primapaste) in foals[116] has produced Fe toxicosis.

(2) Fe injections may be given inappropriately to animals suspected of being Fe-deficient, especially to racehorses.[117] If Fe cannot be excreted fast enough by urine and feces, hyperferremia will develop.

b. Hereditary hemochromatosis was reported in cattle of the Salers breed.[118] 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 (doubling within 2–3 days)[119] and in dogs[2], 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 released Fe may result in increased serum [Fe].
   b. Intravascular and extravascular hemolytic states are associated with increased serum [Fe] because erythrocyte lysis leads to an increase in available Fe that is transported through the plasma for reutilization or storage.
4. False increases
   a. The Fe in free Hgb has very little influence on serum [Fe] because it is not released for detection by most assay methods.
   b. Fe liberated from ferritin can cause increased serum [Fe] when determined by some Fe assays. The amount of increase is minimal unless serum ferritin concentrations are greatly increased.[120]
5. Neonatal foals had serum [Fe] values > 400 μg/dL but the [Fe] decreased to adult values by 3 days of age.[4]

C. Hypoferremia (Table 4.14)

Table 4.14. Disorders and conditions that cause hypoferremia

Disorders and conditions
Fe deficiency
[a]Increased Fe loss: chronic external blood loss
Decreased Fe intake or intestinal absorption
Shift of Fe to storage sites
[a]Acute inflammation
[a]Chronic inflammation
Other or unknown mechanisms
Dexamethasone injections in cattle
Portosystemic shunts in dogs
Young animals (foals, kittens, calves)

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1. Increased Fe loss: chronic external blood loss
   a. Because each milliliter 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.
   b. Causes of blood loss that result in Fe deficiency include intestinal parasitism (hookworms and whipworms in dogs), fleas and ticks (dogs, cats, calves), chronic gastrointestinal hemorrhage due to neoplasia, ulcers, or other lesions, and excessive donation of blood.
   c. Acute blood loss is not expected to cause hypoferremia because Fe is mobilized from storage sites and carried in the plasma by transferrin.
2. Shift of Fe to storage sites
   a. In inflammatory diseases, serum [Fe] is decreased because of the sequestration of Fe in macrophages of liver, spleen, or marrow. IL-1 is involved in the altered Fe kinetics.
   b. The altered Fe kinetics in inflammation that results in hypoferremia has been called a pseudo–Fe deficiency.[121] In this condition, blood may be Fe-deficient but the body is not.
3. Decreased Fe intake or intestinal absorption
   a. Veal calves are purposely fed an Fe-deficient diet that can produce Fe deficiency.
   b. Extensive intestinal mucosal disease that leads to impaired absorption of Fe will contribute to an Fe-deficient state.
4. Glucocorticoids in cattle caused a hypoferremia (from about 140 μg/dL to 50 μg/dL) within one day after dexamethasone (2 mg) was given intravenously.[122]
5. Portosystemic shunts in dogs
   a. Dogs with congenital portosystemic shunts may have a microcytic anemia and some may have a concurrent hypoferremia.[123] In experimental studies, the serum [Fe] did decrease in a group of 16 surgically induced portosystemic shunts (from a mean of 129 μg/dL to a mean of 92 μg/dL), but the individual serum [Fe] remained WRI.[124]
   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.[123]
6. Young animals
   a. Seventy percent of the 2- to 4-week-old kittens in a specific-pathogen-free colony had hypoferremia (relative to adult values) and the hypoferremia was associated with microcytosis.[5] This suggests a transient Fe-deficient state during early growth.
   b. In 18 dairy calves (< 3 days old), serum [Fe] values were lower in calves with Hct values < 25% than those with Hct values > 25%. Only the most severely anemic calf (Hct = 9%) had clinical signs of anemia. The cause of the apparent congenital hypoferremia and Fe-deficient state was not determined.[17]
   c. Relative to adult values, foals have a peak microcytosis between 3 and 5 months of age.[4] However, their serum [Fe] values were equal to or greater than values found in healthy adult horses.[4]

II. TIBC and UIBC
  A. Analytical concepts
    1. Terms and units

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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, serum [Fe] is dependent on and correlates with the transferrin concentration in serum.
b. A transferrin molecule can contain two Fe^{3+} ions, but, in health, Fe^{3+} occupies only about a third of all plasma or serum transferrin Fe-binding sites. UIBC, a measure of the total unused (open) Fe-binding sites on transferrin, is the [Fe] that could be protein-bound in the sample in addition to the [Fe] already present.
c. Unit conversion: μg/dL × 0.01791 μmol/μg × 10dL/L = μmol/L (SI unit, nearest 1 μmol/L)[114]

2. Sample
   a. Serum is preferred.
   b. Serum [TIBC] is stable for a few days if the sample is refrigerated or kept at room temperatures, and it is stable for months if frozen.
3. Principles of serum TIBC and UIBC assays
   a. Transferrin concentrations can be measured directly, but this rarely is done in veterinary medicine.
   b. Typical method
      (1) Excess Fe citrate is added to serum to saturate all Fe-binding sites and then the bound-Fe and free-Fe fractions are separated by chemical methods. The Fe^{3+} in the saturated transferrin molecules reacts with a dye to determine the bound [Fe], which represents the TIBC.
      (2) The UIBC is calculated: UIBC = TIBC – serum [Fe].

B. Increased serum TIBC values (Table 4.15)
   1. Fe deficiency
      a. People with an Fe deficiency may have an increased TIBC because of the increased production of transferrin to carry available Fe to cells.
      b. Fe-deficient dogs typically do not have increased TIBC values.[125]
   2. Young foals, especially near 1 month of age, have much greater TIBC values (> 600 μg/dL) than neonates or adult horses. Colostrum is transferrin-rich but colostral intake does not explain the entire increase in TIBC values during a foal's first month of life.

C. Decreased serum TIBC values (Table 4.16)
   1. Decreased transferrin production

Table 4.15. Disorders and conditions that cause increased TIBC

Increased transferrin production
Fe deficiency: species variable
Other or unknown mechanisms
Young animals (foals)

Table 4.16. Disorders and conditions that cause decreased TIBC values

Decreased transferrin production
*Inflammation
Hepatic insufficiency
Increased transferrin loss (protein-losing nephropathies)

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a. Inflammation: Transferrin is a negative acute phase protein(i.e., its production is decreased by actions of inflammatory mediators such as IL-1).
b. Hepatic insufficiency: Because transferrin is a β-globulin produced by hepatocytes, liver disease that causes hypoproteinemia may cause hypotransferrinemia. Hypotransferrinemia may play a role in the microcytosis that develops in some animals with hepatic insufficiency.
2. Increased transferrin loss: Severe glomerular lesions that result in a protein-losing nephropathy may result in a hypotransferrinemia. The M_r of transferrin is only slightly greater than albumin's.

III. Percent transferrin saturation (% saturation)
A. Analytical concepts
1. Percent transferrin saturation is a calculated value that estimates the percentage of Fe-binding sites on transferrin molecules that are occupied by Fe.
2. Percent transferrin saturation = (serum [Fe] × 100) ÷ TIBC
B. Because the % transferrin saturation is a calculated value, changes depend on changes in the serum [Fe] and TIBC values.
1. Greater % transferrin saturation values occur when serum [Fe] is increased, TIBC is decreased, or both.
2. Lower % transferrin saturation values occur when serum [Fe] is decreased, TIBC is increased, or both.

IV. Stainable Fe in macrophages of marrow, spleen, or liver
A. In microscopic examinations of formalin-fixed tissue or air-dried cytologic preparations, hemosiderin is seen as a yellow to brown granular or globular pigment in macrophages.
B. When attempting to quantify the amount of Fe in storage (especially for Fe deficiency), an Fe-specific stain (such as Prussian Blue) allows 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.
C. The marrow of healthy cats does not contain stainable Fe and thus feline marrow cannot be used to assess Fe stores.

V. Serum ferritin concentrations
A. Analytical concepts
1. Unit conversion: ng/mL × 1000 mL/L × 1 µg/1000 ng = µg/L (SI unit, nearest 10 µg/L)[114]
2. Sample: Serum is preferred. Ferritin concentrations are reported to be stable for 7 days at 2°–8°C, 6 months at -20°C. Repeated thawing and refreezing is not recommended.
3. Principles of serum ferritin assays: Species-specific immunoassays and other immunologic assays are used to measure ferritin in dogs, cats, and horses.[4,126]
B. Hyperferritinemia (Table 4.17)
1. Associated with increased total body Fe
a. If neither inflammation nor hepatic disease is present, serum or plasma ferritin concentrations are highly correlated with Fe storage in people.[127] If Fe toxicity is present, the typical ferritin assay will measure tissue ferritin released from damaged cells and thus the amount of Fe storage will be overestimated.

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Table 4.17. Disorders and conditions that cause hyperferritinemia

Associated with increased total body Fe
Iatrogenic: excess Fe injections or oral hematinics
Genetic defect in regulation of Fe absorption
Increased ferritin production
"Inflammation
Neoplasia: malignant histiocytosis
Shift of ferritin from tissue to plasma
Liver disease
Hemolysis

b. In a group of 95 dogs, there was a significant correlation between serum ferritin concentrations and nonheme-iron content of liver and spleen, but none of the dogs had increased iron stores.[128]

2. Increased ferritin production
   a. Inflammation: Because apoferritin is a positive acute phase protein, cytokines (e.g., IL-1) stimulate hepatocytes to produce more ferritin. In people, the Fe saturation of ferritin decreases with inflammation.[129]
   b. Neoplasia: Hyperferritinemia has been considered a marker for several human neoplasms and may be due to inflammation, accelerated erythrocyte turnover (hemolysis), or increased production by neoplastic cells. Hyperferritinemia has been associated with malignant histiocytosis in dogs and people.
3. Shift of ferritin from tissue to plasma
   a. Liver 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.[127] Most clinical assays probably measure glycosylated and nonglycosylated ferritin.
   b. Hemolytic diseases: Hyperferritinemia may relate to underlying inflammation or to increased transport demands due to Fe recycling from lysed erythrocytes.

C. Hypoferritinemia
   1. Decreased Fe storage
      a. Serum or plasma ferritin concentrations decrease as Fe storage decreases.
      b. In people, ferritin concentrations decrease early in Fe deficiency and before decreased [Hgb], microcytosis, or decreased serum [Fe] is detected. Once Fe storage is depleted, the decreased plasma ferritin concentrations remain relatively constant while hematologic evidence of the deficiency (anemia, microcytosis) develops.[125]
   2. Because serum ferritin concentration may be the net result of opposing processes, an Fe-deficient animal with inflammatory disease may not be hypoferritinemic.

VI. 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 4.18, the two major causes of hypoferremia can be differentiated by the assessment of iron storage(either by stainable marrow or serum ferritin concentrations). However, the other conditions or disorders that can alter serum [Fe], TIBC, and iron storage need to be considered.

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Table 4.18. Comparative Fe profile results

	Serum [Fe]	Serum TIBC	Stainable Fe in marrow[a]	Serum ferritin concentration
Fe deficiency	↓	WRI - ↑	↓	↓
Inflammation	↓	↓	↑	↑
Overload of Fe due to excess intake (diet or iatrogenic)	↑	WRI - ↑	↑	↑
Increased glucocorticoids (except cattle)	↑	?	?	?
Hemolysis	↑	↑	WRI -↑	↑
Young animals (compared to mature)	↓ (foals and kittens)	↑ (foals)	↓	↓
Hepatic insufficiency	?	↓	?	↓ - ↑[b]
Protein-losing nephropathy	?	↓	?	?

[a] Marrow samples of healthy cats do not have stainable Fe.

[b] Could be decreased because of decreased production; could be increased due to hepatocyte damage.

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Chapter 5

HEMOSTASIS

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Table 5.1. Abbreviations and symbols in Chapter 5

»	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
I–XIII	inactive coagulation factors I through XIII (there is no VI); terminal "a" denotes that the factor has been activated (e.g., IIa)
ACT	activated coagulation (clotting) time
ADP	adenosine diphosphate
ATIII	antithrombin III (antithrombin)
ATP	adenosine triphosphate
BMBT	buccal mucosal bleeding time
BVD	bovine virus diarrhea
Ca^{2+}	calcium
DDAVP	1-deamino-8-D-arginine vasopressin
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
ECF	extracellular fluid
EDTA	ethylenediaminetetraacetic acid
EIA	equine infectious anemia
ELISA	enzyme-linked immunosorbent assay
EPI	exocrine pancreatic insufficiency
fCa^{2+}	free ionized calcium
FDP	fibrin or fibrinogen degradation products
FeLV	feline leukemia virus
FIP	feline infectious peritonitis
FIV	feline immunodeficiency virus
GM-CSF	granulocyte/macrophage-colony stimulating factor
GP	glycoprotein
GPIb	glycoprotein Ib
Hct	hematocrit
HMWK	high molecular weight kininogen
IgE	immunoglobulin E
IL-x	interleukin (x for Arabic numbers)
IIHA	idiopathic immune hemolytic anemia
IMT	immune-mediated thrombocytopenia
INR	international normalized ratio
ISI	international sensitivity index
MPS	mononuclear phagocyte system
MPV	mean platelet volume
mRNA	messenger ribonucleic acid
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PCR	polymerase chain reaction
PDW	platelet distribution width
PF3	platelet factor 3
PGI_2	prostacyclin
PIVKA	proteins induced by vitamin K antagonism or absence
protein Ca	activated protein C

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PK	prekallikrein
PSAIg	platelet surface-associated immunoglobulin
PT	prothrombin time
PTT	activated partial thromboplastin time
QBC	quantitative buffy coat
RMSF	Rocky mountain spotted fever
RVVT	Russell viper venom time
SI	Système International d'Unités
SLE	systemic lupus erythematosis
TAT	thrombin-antithrombin complexes
TF	tissue factor
TFPI	tissue factor pathway inhibitor
t-PA	tissue-type plasminogen activator
TT	thrombin time
TT_{Clauss}	modified TT for [fibrinogen]
TxA_2	thromboxane
vWD	von Willebrand disease
vWf	von Willebrand factor
vWf:Ag	von Willebrand factor as detected antigenically
WBCT	whole blood clotting time
WRI	within reference interval

HEMOSTASIS

I. Hemostasis is the arrest of bleeding or the interruption of blood flow through a vessel. The term *hemostasis* 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 interactions of platelets, coagulation factors, fibrinolytic factors, and blood vessels (Fig. 5.1).

II. Abnormal hemostasis may result in hemorrhage or thrombosis. Laboratory testing of the individual components of the hemostatic system may be used to discover, explain, monitor, or prognosticate these pathologic states.

PLATELETS (THROMBOCYTES)

I. Physiologic processes
   A. Platelets are produced from megakaryocytes (*mega* = large, *karyon* = nucleus, *cyte* = cell) that are derived from pluripotent hematopoietic stem cells (see Fig. 3.1).
      1. Megakaryocytopoiesis, the proliferation and maturation of megakaryocytes, occurs in hematopoietic tissue, mostly bone marrow. Resident myeloid progenitor cells respond to cytokines, primarily thrombopoietin, by undergoing proliferation and maturation.[1] Limited baseline megakaryocyte production occurs in the absence of thrombopoietin.[2]
      2. Thrombopoiesis, the formation of platelets from megakaryocytes and their delivery to the circulation, is also primarily mediated by thrombopoietin,[1] but baseline platelet production can occur in the absence of thrombopoietin.[2] Thrombopoiesis occurs in

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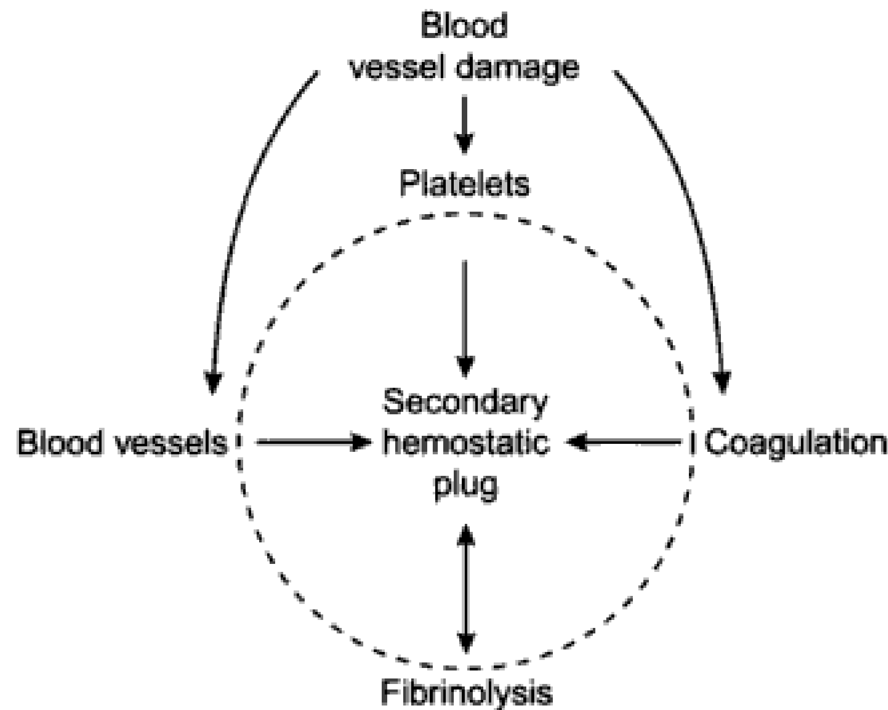

Fig. 5.1. Normal hemostasis is maintained by the numerous and complex interactions of blood vessels, platelets, coagulation pathways, and the fibrinolytic system. When blood vessel damage occurs:

- Vasoconstriction reduces blood loss, and activated endothelial cells express both prothrombotic functions to limit bleeding and antithrombotic functions to limit clotting.
- Platelets adhere to exposed subendothelium, spread to patch the defect, release products that activate other platelets, and aggregate together to form a primary hemostatic plug. Their secretory products also help maintain vasoconstriction, and their membranes are an important source of phospholipid to accelerate coagulation.
- The intrinsic and extrinsic coagulation pathways are activated, leading to the production of thrombin and subsequent conversion of fibrinogen to fibrin within the primary hemostatic plug. This forms a stable secondary hemostatic plug that controls bleeding. Thrombin also activates platelets and endothelial cells.
- When coagulation pathways are activated, so is fibrinolysis. Fibrinolysis helps control the extent of coagulation by breaking down fibrin, thus contributing to the formation of an appropriate secondary hemostatic plug and promoting eventual removal of the plug to maintain normal blood flow.
- Abnormalities of any component of this system can upset the balance and lead to either hemorrhage or thrombosis.

the bone marrow and at other sites of hematopoiesis. It also occurs in the lungs, where megakaryocytes lodge after circulating from the bone marrow (see Plate 4.*A*).[3] Platelets form from late-stage megakaryocytes. They appear to shed directly into the blood by cytoplasmic fragmentation or by the periodic constriction of megakaryocytic cytoplasmic pseudopodia that extend into vascular spaces.

3. The sites of thrombopoietin production during health and disease are not completely clear, but mRNA for thrombopoietin is expressed in several tissues, including hepatocytes, renal tubular epithelium, and stromal cells of the bone marrow.[1] There is evidence that production rates are relatively constant and that plasma thrombopoietin is cleared by platelets[4] and megakaryocytes[5] after it binds to their membrane receptors. Platelet and megakaryocyte masses therefore seem to control plasma thrombopoietin concentrations. With decreased platelet mass, more thrombopoietin remains unbound and is available to stimulate megakaryocytes. As the platelet concentration increases, more thrombopoietin is bound and removed from circulation so there is less stimulation of megakaryocytes or other stem cells. Activated platelets may release intact thrombopoietin *in vivo*, thus increasing blood concentrations during platelet consumptive states.[6]

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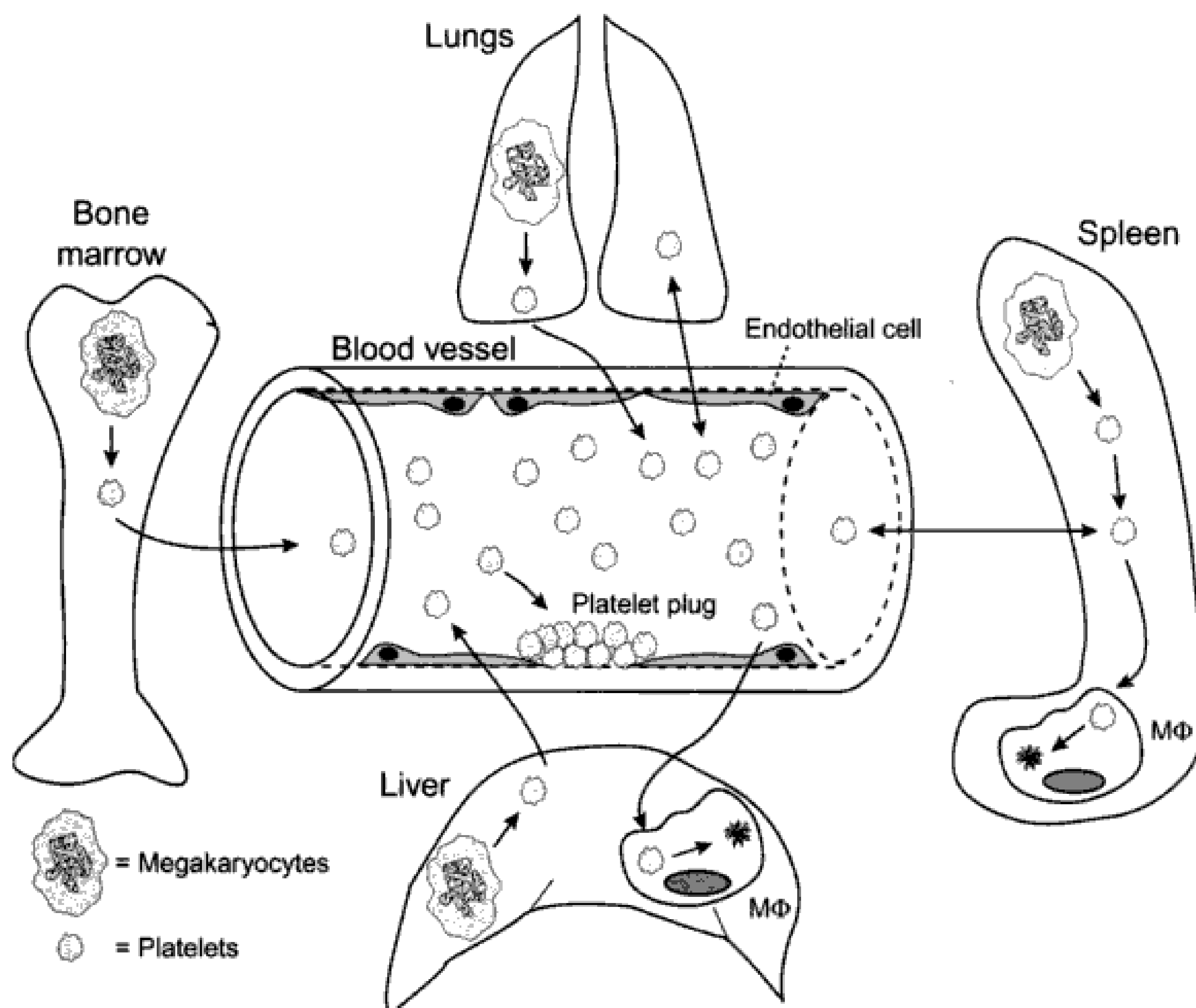

Fig. 5.2. Blood platelet concentrations are established by the relative rates of platelet production, consumption, and destruction and by the shifting of platelets to and from the circulation. (MΦ = macrophage)

- *Production:* Most megakaryocytopoiesis and thrombopoiesis occur in the bone marrow, and new platelets enter systemic circulation via marrow sinusoids. Some megakaryocytes appear to become lodged in the lungs after release from the bone marrow. These megakaryocytes release platelets into the blood, but the overall importance of this process is not known. Platelets produced by splenic hematopoietic tissue may contribute to the circulating platelet mass in health and disease. Hematopoietic foci may also arise in the liver and contribute to the circulating platelet mass.
- *Consumption:* Platelets may be removed from circulation during normal maintenance of vascular integrity or during accelerated consumptive states (e.g., thrombotic disease, vasculitis).
- *Destruction:* Macrophages, primarily in the spleen and liver, may destroy platelets that carry surface-associated antibodies or complement. Aged or damaged platelets may be similarly destroyed.
- *Redistribution:* Splenic platelet sequestration may reduce the circulating platelet mass, and splenic contraction may increase it. Pulmonary sequestration of platelets has been associated with severe hypothermia and endotoxemia.

B. Platelet kinetics

1. Blood platelet concentrations are established by the relative rates of platelet production, consumption, and destruction, and by the shifting of platelets to and from the circulation (Fig. 5.2).
2. 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 to 10 days.[7]

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3. Platelet consumption is ongoing. Mean platelet life span appears to be decreased in any markedly thrombocytopenic state, even when thrombocytopenia is due to marrow hypoplasia. This can be explained by a fixed platelet requirement for maintenance of vascular integrity, so when marked thrombocytopenia is present, a substantially greater percentage of the circulating platelets are consumed during routine maintenance.[8]
4. Factors governing platelet life span are unclear, but the spleen is important in determining platelet life span in dogs. Splenectomized dogs had almost 50% longer platelet life spans (8 days) than healthy nonsplenectomized dogs (about 5.5–6 days).[9] Platelet life spans are similar in cattle and swine.[10,11]
5. Human and rabbit spleens have been shown to harbor about 33% of blood platelets at any given moment.[7,12,13] Epinephrine and splenic contraction can mobilize these platelets to circulation, while splenic engorgement can trap more platelets there.[7,13] Splenic pooling and mobilization of platelets is expected in other species too (e.g., dogs).[13]

C. Platelet functions
1. Platelets are required for formation of primary hemostatic plugs to repair small vascular defects, and they amplify minute stimuli into explosive production of fibrin to form secondary hemostatic plugs. Platelet hemostatic functions can be divided into five major categories: adhesion, aggregation, release (secretion), facilitation of coagulation, and clot retraction.
   a. Adhesion: Platelets adhere to and spread over (patch) perturbed endothelium or exposed subendothelium, mostly via vWf that binds to platelet GPIb.
   b. Aggregation: When adherence or platelet agonists (e.g., ADP, collagen, PAF, thrombin) activate platelet membrane $\alpha_{IIb}\beta_3$ receptors, platelets aggregate via fibrinogen or vWf bridges that bind to $\alpha_{IIb}\beta_3$ on neighboring platelets.
   c. Release (secretion): Activated platelets can release preformed granular contents (e.g., fibrinogen, Factor V, ADP, ATP, plasminogen) and newly formed mediators such as TxA_2 and arachidonic acid. These products help mediate the hemostatic process.
   d. Facilitation of coagulation: When platelets are stimulated, anionic membrane phospholipids that can support coagulation move from the inner to the outer membrane, where they are available for use as cofactors in the coagulation pathways. These phospholipids include phosphatidylserine and are referred to as PF3.[14,15] Platelets provide specific high-affinity binding sites for coagulation enzymes, cofactors, and zymogens. Platelets also release platelet agonists that activate other platelets.
   e. Clot retraction: Most analogous to muscle contraction, since it involves platelet actin and myosin; it facilitates wound closure and vessel patency.
2. Energy for platelet functions is derived mostly from ATP generated by glycolysis. Mitochondria are few and small, so oxidative metabolism is minimal. Glucose enters platelets by facilitated diffusion.
3. Nonhemostatic platelet functions: Platelets, through their release of mediators and interaction with leukocytes, are important in inflammation and wound healing.

II. Morphologic features of platelets (see Plate 4)
A. Platelets are anucleate disk-shaped cytoplasmic fragments of megakaryocytes.
B. In blood films, platelets are present individually or in small to large clumps (see Plate

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4.*B*, *C*, and *D*). Clumps usually result from *in vitro* platelet activation, but anticoagulant-induced agglutination of nonhuman platelets has been reported.[16] Clumps are most frequent in feline and bovine samples. They may be found throughout a blood film, but they are best found at its feathered edge, sides, and base.

C. Shape: Nonactivated platelets are round, oval, or elongate. Elongate forms probably represent undivided megakaryocyte pseudopod segments (proplatelets); they may be more numerous during stimulated thrombopoiesis (see Plate 4.*L* and *M*). Activated platelets may have peripheral pseudopodia (see Plate 4.*H*, *I*, and *J*).

D. Granules: With Wright-staining, nonactivated platelets have clear to pale blue cytoplasms containing small pink to purple granules. With activation, the granules may be centralized (see Plate 4.*I* and *J*) or absent (secreted) (see Plate 4.*H*). Granules tend to be most prominent in feline and bovine platelets and least prominent in equine platelets.

E. Size: Platelets in most healthy individuals are fairly uniform in size. The diameter of feline platelets, which is greater and more variable than in other species, may exceed that of feline erythrocytes.[17]

F. *Ehrlichia platys* morulae may be present in platelets (see Plate 4.*Q*). They must be differentiated from stain precipitate and fragments of nuclear material (see Plate 4.*S*).

III. Platelet concentration (often called "platelet count" in clinical jargon)

A. Analytical concepts (see Chap. 2 for more details)

1. Units: thousands/µL of blood; $300 \times 10^3/\mu L = 300 \times 10^9/L$ (SI unit; suggest reporting to nearest $10 \times 10^9/L$)

2. Sample

a. Platelet concentrations are reportedly stable for 5 hr at room temperature and 24 hr when samples are refrigerated (4°C).[18] On the average, platelet concentrations remained relatively stable in EDTA-anticoagulated canine blood for 48 hr (kept at room temperature for the first 8 hr and refrigerated for the following 40 hr), but there were considerable increases and decreases in platelet concentrations of individual samples.[19]

b. EDTA anticoagulation is routine, but citrated blood (1 part citrate to 9 parts blood) can be used (e.g., when EDTA-induced platelet clumping is suspected). Citrate dilution requires that the measured platelet concentration be corrected (corrected platelet concentration = measured platelet concentration $\times$ 1.1). Some commercial citrate tubes contain platelet inhibitory factors that help reduce *in vitro* platelet activation.[20] Platelet clumping is frequent with heparinized samples.

3. Methods

a. For all platelet quantitation methods, platelet clumping must be absent from blood smears to be confident that a platelet concentration is accurate.

b. Most analyzers detect and enumerate platelets by impedance or light scatter methods (see Chap. 2). Light scatter methods allow better differentiation of large platelets from erythrocytes.

c. The QBC hematology analyzer actually measures a "thrombocrit," the percentage of the volume of blood occupied by platelets. Because the value is affected by platelet size, it may not always match platelet concentrations based on other methods. However, compared to platelet concentration, thrombocrit may be a better reflection of the total platelet mass, platelet functional potential, and thrombopoietic stimulus.[21] Also, the analytical method is less affected by small platelet clumps.[22]

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B. Appropriate reference intervals for platelet concentration may be shifted upward for individuals at high altitudes.[23]

IV. Thrombocytopenia

A. General concepts

1. Definition: Platelet concentration is less than a valid lower reference limit (platelet clumps must be absent); healthy greyhounds[24,25] and perhaps shibas[26] have lower platelet concentrations than other dogs.
2. Thrombocytopenia is a pathologic state or diagnostic problem, not a disease. Its major significance is the potentiation of bleeding when platelet concentrations are markedly decreased. It is also useful to recognize because its presence may suggest specific pathologic states or diseases.
3. Clinical signs: Petechiae and ecchymoses are the hallmarks of severe thrombocytopenia. Mucosal bleeding (epistaxis, hematochezia, melena, hematuria, hyphema) and prolonged hemorrhage after accidental or intentional trauma (e.g., venipuncture) are common.
4. Thrombocytopenia can occur in many diseases,[27-30] but there are only three major pathogenic mechanisms to be considered: abnormal platelet distribution, decreased platelet production, and decreased platelet survival. Iatrogenic platelet dilution may also cause or contribute to thrombocytopenia. Combinations are common.

B. Diseases and conditions (Table 5.2)

1. Pseudothrombocytopenia is false thrombocytopenia that may occur when not all the platelets in a sample are counted. It can be recognized when microscopic examination reveals platelet clumps or yields an estimate of platelet concentration that is greater than the measured platelet concentration.
   a. Pseudothrombocytopenia usually occurs because blood collection causes platelet activation and aggregation, so platelets are clumped and not counted individually.
   b. It also occurs with automated analyzers when many large platelets are present but not detected because they exceed the upper size limit of detection; for such samples, platelet concentrations should be determined by manual methods.
   c. It can occur with cold agglutinins or anticoagulant-induced, antibody-mediated agglutination of platelets; anticoagulants, especially EDTA, can unmask antigenic sites on platelet membranes so that immunoglobulins bind and bridge platelets together.[31] Although rarely reported in veterinary species,[32] it is quite common with human samples.
2. Abnormal platelet distribution (sequestration)
   a. 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.
   b. The term *sequestration* is often used for either reversible platelet redistribution or for irreversible trapping of platelets in organs due to destruction by the MPS. However, the latter process should fall under the category of decreased platelet survival.
   c. Abnormal platelet distribution may contribute to or (rarely) cause marked thrombocytopenia.
   d. Disorders
      (1) Splenomegaly appears to cause splenic pooling of an exchangeable platelet

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Table 5.2. Diseases and conditions that cause thrombocytopenia

- Abnormal platelet distribution (sequestration): splenomegaly, severe hypothermia, endotoxemia
- Decreased platelet production (myelosuppression of one to all hemic cell lines)
  - Drugs (toxicants)
    - [a]Predictable: chemotherapeutic agents, estrogens in dogs (exogenous, endogenous), bracken fern poisoning (ruminants)
    - Idiosyncratic: phenylbutazone, meclofenamic acid, trimethoprim-sulfadiazine, trimethoprim-sulfonamide, griseofulvin
  - Immune-mediated: amegakaryocytic thrombocytopenia, impaired thrombopoiesis
  - Infectious (usually multifactorial; see text)
  - Irradiation (whole body or extensive): prior to autologous marrow transplantation
  - Marrow replacement: bone marrow neoplasia (primary hemic or metastatic), myelofibrosis, osteopetrosis
  - Megakaryocytic leukemia (M7)
  - Myelonecrosis: infections, neoplasia, toxicants
- Decreased platelet survival (accelerated platelet destruction and consumption)
  - Immunologic
    - [a]Primary (idiopathic) IMT
    - Secondary IMT
      - Drug-induced: gold salts, sulfonamides
      - Infectious (usually multifactorial; see text)
      - Neonatal alloimmune thrombocytopenia
      - Neoplasia (usually multifactorial; see text)
      - Posttransfusion purpura
      - Systemic immune-mediated disease: SLE, Evans' syndrome
  - Nonimmunologic
    - Blood loss, acute and severe: anticoagulant rodenticides, blood loss with ECF volume replacement
    - Platelet activation with accelerated consumption or utilization
      - Localized intravascular coagulation: hemangiosarcoma, hemorrhage, thrombosis
      - [a]DIC: envenomation, hepatic disease, infections, massive necrosis, pancreatitis, neoplasia, overheating, septicemia
      - Drugs: protamine sulfate
      - Envenomation (without DIC)
      - [a]Vasculitis: RMSF, canine herpes virus infection, hemolytic uremic syndrome, dirofilariasis, cutaneous and renal glomerular vasculopathy of greyhounds
- Hemodilution: infusion with colloids, crystalloids, plasma
- Idiopathic mechanism or multifactorial (decreased production and decreased survival)
  - Anaphylaxis
  - [a]Infections: babesiosis, BVD, canine distemper, canine parvoviral diarrhea, cytauxzoonosis, ehrlichiosis, EIA, endotoxemia, FeLV, FIP, histoplasmosis, leishmaniasis, leptospirosis
  - [a]Neoplasia: carcinomas, hemangiosarcoma and other sarcomas, lymphoma, leukemias
  - Drugs
  - Hypophosphatemia associated with hyperalimentation

Notes: Lists of specific disorders or conditions are not complete but are provided to give examples.

Platelet concentrations in greyhound dogs are lower than for other breeds of dogs; pseudothrombocytopenia caused by platelet clumping or giant platelets (e.g., Cavalier King Charles spaniels) should be considered before pursuing causes of true thrombocytopenia.

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fraction in some human conditions, though platelet survivals may also be decreased.[33] Similar effects of splenomegaly have been presumed to occur in other species.

(2) Severe hypothermia (rectal temperatures of 20°C) may cause reversible platelet redistribution to liver and spleen in dogs.[34]

(3) Endotoxemia has been associated experimentally with transient pulmonary platelet pooling and subsequent hepatic sequestration and probable destruction in dogs.[35,36]

3. Decreased platelet production
   a. Adequate platelet production requires a healthy megakaryocyte population. Megakaryocytes can be unhealthy because of generalized bone marrow diseases or because of megakaryocyte-specific diseases that either diminish megakaryocytopoiesis or impair thrombopoiesis. Unexplained bicytopenia and pancytopenia suggest the possibility of generalized bone marrow disease.
   b. Recognized conditions associated with production failure thrombocytopenias in animals are acquired.
      (1) Drugs (toxicants)
         (a) Predictable and dose-dependent myelosuppressive effects (examples)
            (i) Antineoplastic chemotherapeutic agents, including alkylating agents, antimetabolites, and antibiotics (e.g., doxorubicin), frequently induce thrombocytopenia 1–2 weeks after drug administration.[37,38]
            (ii) Estrogens from exogenous sources[39] or endogenous sources (e.g., testicular Sertoli cell neoplasm,[40] rare ovarian granulosa cell neoplasms,[41] and rare interstitial cell neoplasms[42] 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.[43]
            (iii) Bracken fern poisoning may induce aplastic anemia with thrombocytopenia in ruminants,[44] and albendazole has been implicated in a dog.[45]
         (b) Drugs (toxicants) with idiosyncratic (i.e., sporadic and unpredictable) myelosuppressive effects (examples)
            (i) Idiosyncratic myelosuppression is suspected much more often than it is proven; relapse with re-challenge after recovery would be supportive of a cause-and-effect relationship, but it is avoided.
            (ii) Phenylbutazone and meclofenamic acid are nonsteroidal anti-inflammatory drugs that have been associated with bone marrow hypoplasia and bicytopenia or pancytopenia in dogs and probably in horses (only phenylbutazone).[46-48]
            (iii) Trimethoprim-sulfadiazine and trimethoprin-sulfonamide have been associated with aplastic anemia in dogs and cats.[49-51] Its 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 in affected dogs. Thrombocytopenia caused by myelosuppression should be differenti-

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ated from immune-mediated platelet destruction caused by trimethoprim-sulfa-dependent antibodies.
(iv) Griseofulvin has caused marrow suppression in cats,[52,53] and albendazole has been implicated in a cat.[45]

(2) Immune-mediated suppression of megakaryocytopoiesis
  (a) Suspected amegakaryocytic thrombocytopenia has been rarely reported in dogs and cats.[54,55] In people, it may occur with antibody- or cell-mediated[56] destruction of megakaryocytes or their precursors, or with antibody-mediated destruction of required cytokines.[57,58]
  (b) Antimegakaryocyte antibodies may also lead to impaired thrombopoiesis[59] 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.[60]

(3) Infections: The pathogenesis of thrombocytopenia associated with many infections is multifactorial, often including decreased platelet production. Several mechanisms of decreased production may be involved.
  (a) Direct infection of megakaryocytes or other hematopoietic precursors may affect megakaryocytopoiesis or thrombopoiesis (e.g., BVD,[61] canine distemper[62]).
  (b) Myelosuppressive cytokines may be produced in response to infection (e.g., EIA).[63,64]
  (c) Incompletely defined mechanisms (e.g., chronic canine monocytic ehrlichiosis, canine parvoviral infection); in addition to the effects of septicemia/endotoxemia associated with severe enteritis, 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, it was not in recognizable cells of the megakaryocyte series.[65,66]

(4) Irradiation (whole body or extensive): Whole body irradiation 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.[67,68]

(5) Marrow replacement (myelophthisis): Bone marrow neoplasia (primary hemic or metastatic), myelofibrosis, and osteopetrosis may each induce myelosuppression. Mechanisms may include physical replacement of normal cell populations, competition for nutrients, obstruction of blood supply, lysis of marrow cells, or secretion of inhibitors.

(6) Megakaryocytic leukemia is very rare but may be associated with thrombocytopenia,[69,70] probably because of dysthrombopoiesis and decreased thrombopoiesis.

(7) Myelonecrosis (with or without myelofibrosis): Infections, neoplasia, or toxicants may cause enough marrow necrosis to contribute to thrombocytopenia.[71,72]

4. Decreased platelet survival (accelerated platelet destruction and consumption)

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a. If increased platelet production does not compensate for accelerated platelet destruction or consumption, thrombocytopenia will ensue. Platelet "consumption" usually refers to the utilization of platelets during hemostatic functions, whether physiologic or pathologic. Platelet "destruction" usually refers to the death of platelets occurring by direct damage, usually immune-mediated phagocytosis.
b. Immunologic causes of decreased platelet survival
   (1) General concepts: IMT usually occurs when an animal's immune system produces antibodies that bind directly or indirectly to its own platelets (PSAIg). This leads to accelerated platelet destruction by the MPS. Destruction is mediated by PSAIg, platelet surface-associated complement, or both. The process may be initiated by a defective immune system, defective platelets, or adsorbed antigens from drugs, infectious agents, or neoplasms. The cause is usually unknown.
      (a) Classification of thrombocytopenia as immune-mediated is often done clinically based on clinical findings and response to immunosuppressive therapy. Detection of increased PSAIg by a validated direct assay would provide more support for the conclusion.
      (b) The abbreviation ITP is also used for IMT. 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. Purpura may not be present in patients with IMT. Idiopathic thrombocytopenia may not always be immune-mediated.
      (c) IMT not associated with a detected disease or condition is idiopathic IMT, often called primary IMT. It may be autoimmune, but antibody specificity to normal self-epitopes is rarely documented in veterinary medicine.
      (d) IMT that is part of a more widespread disease (e.g., SLE) is often called secondary IMT. IMT occurring in association with other conditions has also been considered secondary IMT. Secondary IMT may (e.g., SLE) or may not have an autoimmune pathogenesis.
   (2) Idiopathic IMT (primary, often presumed to be autoimmune)
      (a) Idiopathic IMT is well documented and relatively common only in dogs and people, but it appears to occur in other species as well.[73-77] It is primarily a diagnosis of exclusion; increased PSAIg or positive megakaryocyte immunofluorescence results may be supportive (see below). If associated diseases have been carefully excluded before labeling as idiopathic, an autoimmune pathogenesis is likely. Immunoblotting and immunoprecipitation studies have confirmed a role for platelet membrane antigen targets in at least some cases of canine idiopathic IMT.[78]
      (b) Common laboratory features:[79] Thrombocytopenia (usually < 50,000/µL, often < 10,000/µL); MPV increased, decreased, or WRI; anemia (about 50% of cases); megakaryocytic hyperplasia (megakaryocytic hypoplasia can occur)
   (3) Secondary IMT
      (a) Drug-induced IMT
         (i) Exposure to certain drugs may lead to increased PSAIg and therefore accelerated platelet destruction because of the production of drug-

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dependent or drug-independent antibodies. 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. Drug-dependent antibodies are more common in people,[80] and they can theoretically bind to any of the following: a drug or a metabolite adsorbed to the platelet surface, a platelet epitope that is cross-reactive with the drug or a metabolite, a platelet surface antigen exposed by the presence of the drug or a metabolite, or a combined drug-platelet neoantigen on the platelet membrane.

(ii) Drug-induced IMT is suspected when otherwise unexplained thrombocytopenia, usually severe, develops a few days after drug exposure, and when the thrombocytopenia resolves rapidly after drug withdrawal.

(iii) Strict criteria to be met for confirmation of drug-induced IMT by drug-dependent antibodies are (1) increased PSAIg in a patient that developed thrombocytopenia at least a few days after drug exposure; (2) 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; (3) resolution of both thrombocytopenia and increased PSAIg after drug withdrawal; and (4) relapse after reexposure to the drug (not recommended).

(iv) Numerous drugs have been suspected to cause IMT in dogs, but gold salts (auranofin, gold sodium thiomalate, and possibly aurothioglucose)[81] and sulfonamides (with or without trimethoprim)[82,83] have been strongly implicated; platelet antibody assay results have been supportive, but the assays used had limited validation. Methimazole and propylthiouracil have been incriminated in cats.[84,85] 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, but not all species express them.[86]

(b) IMT associated with infection (usually multifactorial; see below)

(i) Pathogeneses of thrombocytopenias associated with infection are usually multifactorial or unknown, but many bacterial, viral, fungal, and protozoal diseases have been associated with IMT in people, and similar associations are likely in domestic mammals.

(ii) Infection-associated IMT may result from cross-reactive antibodies, bacteria-induced production of autoantibodies, exposure of otherwise hidden platelet membrane antigens by the organism, binding of antiorganism antibodies to the infectious agent attached to the platelet membrane, or the induction of immune complexes that adhere to the platelet membrane.

(iii) May occur with acute canine ehrlichiosis,[87] RMSF,[88] histoplasmosis,[89] leishmaniasis,[90] distemper or modified live virus distemper vaccination,[62] and EIA[91]

(c) Neonatal alloimmune thrombocytopenia

(i) Thrombocytopenia occurs when passively acquired (mainly via colostrum) maternal alloantibodies to paternal epitopes on a

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neonate's platelets circulate in the neonate's blood and cause 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. It has been described in horses and pigs.[92,93]

(ii) It must be differentiated from other causes of thrombocytopenia in the neonate, especially sepsis. Confirmation entails detection of increased PSAIg in the neonate but not in the dam, and showing the presence of antibodies in the dam's blood that react with paternal and neonatal, but not maternal, platelets. Validated tests may be unavailable.

(d) Neoplasia (usually multifactorial; see below)

(i) Thrombocytopenia occurs commonly in animals with neoplasia. The cause is often multifactorial but may include immunologic mechanisms.

(ii) 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.[94] IMT has been associated with lymphoma in dogs and horses.[77,95]

(e) Posttransfusion purpura

(i) A condition in human transfusion recipients occurring about 1 week after transfusion; associated with severe thrombocytopenia and high titers of platelet-reactive alloantibodies that mediate the destruction of transfused as well as the patient's own platelets

(ii) Rarely, posttransfusion thrombocytopenia has been reported in dogs, but the pathogeneses have not been established.[96]

(f) Systemic immune-mediated disease: Systemic immune-mediated diseases such as SLE are caused by general B-cell activation and the production of autoantibodies directed against multiple targets, one of which may be platelets.

(i) SLE: Though rarely documented, there is evidence of increased PSAIg in dogs with SLE.[79] Primary IMT and SLE may be different disorders within a spectrum of autoimmune diseases.

(ii) Evans' syndrome

- This syndrome was first described in people as idiopathic immune hemolytic anemia and concurrent thrombocytopenia or neutropenia,[97] but the term is now used to refer to concurrent IMT and IIHA.
- In at least some human patients, the antibodies mediating the two cytopenias are distinct.[98] Information is unavailable for animals.
- IIHA with concurrent presumed IMT occurs in dogs (first reported in 1965[99]). An immune pathogenesis has been implicated in some cases by indirect assays for platelet- or megakaryocyte-reactive antibodies in patient plasma.[79]

c. Nonimmunologic causes of decreased platelet survival

(1) Blood loss, acute and severe

(a) When bleeding accompanies severe thrombocytopenia, hemorrhage is probably secondary to the thrombocytopenia rather than thrombocytopenia being caused by the bleeding.

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(b) In dogs, experimental acute, severe blood loss via phlebotomy caused mild to moderate thrombocytopenia (up to 50% reduction in platelet concentration).[100-102]

(c) Thrombocytopenia may be present in bleeding dogs with anticoagulant rodenticide toxicosis.[103] Platelets are probably consumed at an accelerated rate by utilization at sites of hemorrhage throughout the body. With extensive hemorrhage, loss of platelets contributes to thrombocytopenia. Thrombocytopenia may become severe during treatment of these dogs with fluids, transfusions, and vitamin K_1; blood dilution may be contributory.

(2) Platelet activation with accelerated consumption or utilization: Any disorder associated with increased platelet activation may accelerate platelet consumption and lead to thrombocytopenia if platelet production does not compensate.

(a) Localized (controlled) intravascular coagulation (e.g., hemangiosarcoma, thrombosis, hemorrhage) may be associated with platelet consumption at the site(s) where coagulation occurs. Activation of the coagulation cascade leads to the generation of thrombin (Factor IIa), a potent platelet activator. Thrombin activates platelets, and activated platelets release platelet-activating substances that recruit and consume more platelets. Thrombocytopenia may ensue.[104]

(b) DIC: Widespread, uncontrolled activation of the coagulation cascade may produce thrombocytopenia in the same way as localized coagulation, but the likelihood of thrombocytopenia is greater. DIC may result from envenomation, hepatic disease, infections, massive necrosis, pancreatitis, neoplasia, overheating, or septicemia.

(c) Drugs and foreign materials: Some drugs cause or contribute to thrombocytopenia by directly activating platelets. Protamine sulfate, used to reverse the effects of heparin, can induce severe thrombocytopenia in heparinized and nonheparinized dogs, apparently through a direct proaggregatory effect.[105] Foreign materials used within the vascular system (e.g., tubing, catheters) are tested and chosen to minimize platelet activation, but accelerated consumption may occur.

(d) Envenomation (without DIC): There are numerous types of venoms from numerous animal species, and they differ in their effects. Venom from some snakes may contain platelet-activating factors that induce thrombocytopenia in the absence of DIC.[106] Other venoms induce DIC.[107]

(e) Vasculitis: Inflammation of blood vessels 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 results from infectious, immune-mediated, and chemical causes. Infectious causes include RMSF, canine herpes virus infection, dirofilariasis, infectious canine hepatitis, and equine viral arteritis. Endothelial cell damage and necrosis lead to thrombocytopenia without DIC in dogs with hemolytic uremic syndrome or similar disorders (e.g., cutaneous and renal glomerular vasculopathy of greyhounds).[108-110] When evaluating greyhounds, it is important to recognize that their platelet concentrations are lower than commonly reported canine reference intervals.

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5. Hemodilution: Massive dilution of blood with platelet-poor fluids (e.g., crystalloids, colloids, plasma) may cause mild to moderate decreases in blood platelet concentration.[111]
6. Idiopathic mechanism or multifactorial (i.e., decreased production and decreased survival)
   a. The specific mechanisms by which thrombocytopenia occurs are unknown and probably multifactorial for many diseases including infectious, neoplastic, and drug-induced conditions. Even when severe thrombocytopenia results from production failure, there is decreased platelet survival. This occurs because the platelets that do circulate are consumed at an accelerated rate; a greater percentage of them than normal are used for routine maintenance of vascular integrity. In IMT, although platelet destruction is usually instrumental in causing thrombocytopenia, platelet production may also be impaired by immunologic mechanisms.
   b. Anaphylaxis (type I hypersensitivity)
      (1) Anaphylaxis 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.
      (2) Anaphylactic thrombocytopenia may result from inflammatory mediators, DIC, or immune-complex interactions with platelets. Membrane receptors for IgE exist on a subpopulation of human platelets, and they mediate inflammatory platelet reactions in some species.[112]
   c. Infections
      (1) Thrombocytopenia is commonly associated with infections;[27,28,30] certain bacterial (*Ehrlichia* sp.), fungal (e.g., *Histoplasma* sp.), viral (e.g., equine infectious anemia virus), and protozoal (e.g., *Leishmania* sp., *Babesia* sp.) organisms often induce thrombocytopenia. Thrombocytopenia occurs somewhat less frequently, but not unexpectedly, with other organisms (e.g., leptospirosis, feline immunodeficiency virus).[113]
      (2) Infectious thrombocytopenia may be caused by various combinations of suppressed platelet production (direct infection, immune suppression, local effects of inflammation within the bone marrow), altered platelet distribution, increased platelet consumption, and immune-mediated or nonimmune platelet destruction.
   d. Neoplasms[114]
      (1) Many types of neoplasia are associated with thrombocytopenia, including carcinomas, sarcomas, lymphomas, and leukemias. In one study, approximately 10% of dogs with neoplasia were thrombocytopenic, though sometimes it was because of concurrent infections or therapy.[115]
      (2) Thrombocytopenia may result from many mechanisms, alone or combined. In specific cases, causes are usually speculative. Potential mechanisms:
         (a) Decreased production
            (i) Myelophthisis: presumed contributor to thrombocytopenia in dogs with multiple myeloma, acute leukemia, chronic lymphocytic leukemia; other cytopenias sometimes present
            (ii) Myelodysplasia
            (iii) Estrogen secretion by the neoplasm
            (iv) Chemotherapy

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(b) Decreased platelet survival
    (i) Most evaluated dogs with neoplasia and decreased platelet survival (kinetic studies) had hyperfibrinogenemia and fibrinogen half-lives were not decreased.[116,117] This does not support DIC. However, DIC does occur in some cancer patients and may contribute to thrombocytopenia seen in dogs with mast cell neoplasia or hepatic metastases.[115,118] Vasculitis or thrombosis within a neoplasm (e.g., hemangiosarcoma, a malignancy of endothelial cells) may accelerate platelet consumption. Secondary IMT may also occur and has been incriminated in dogs with certain types of neoplasia (e.g., lymphoma) using indirect platelet and megakaryocyte assays.[79]
    (ii) Other contributors may include hemorrhage secondary to a neoplasm, sepsis secondary to immunosuppression, or destruction (phagocytosis) by neoplastic macrophages (e.g., malignant histiocytic neoplasia).
(c) Abnormal platelet distribution: Splenomegaly or hepatomegaly induced by neoplastic infiltration (e.g., hemangiosarcoma) or secondary organ congestion may be associated with platelet sequestration.

e. Drugs: 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 thrombocytopenia is not clear.[119]

f. Hypophosphatemia: Hypophosphatemia resulting from hyperalimentation in *starved* dogs caused decreased platelet survival and thrombocytopenia.[120] Thrombocytopenia was associated with decreased platelet ATP concentrations, probably due to an associated decrease in anaerobic glycolysis. The specific mechanism of accelerated platelet clearance was not determined. Cats receiving total parenteral nutrition also developed thrombocytopenia, but they were not hypophosphatemic and the cause was not apparent.[121]

V. Thrombocytosis

A. General concepts

1. Definition: platelet concentration greater than a valid upper reference limit
2. Thrombocytosis may result from redistribution or increased production of platelets. Increased production may be associated with hemic neoplasia involving megakaryocytes, or it may occur as a secondary reaction to other conditions.
3. Serum [K^+] may become increased *in vitro* (pseudohyperkalemia) by the increased amount of platelet K^+ released during clotting.

B. Diseases and conditions (Table 5.3)

1. Hemic neoplasia (clonal thrombocytosis)
    a. Primary (essential) thrombocythemia is a rare myeloproliferative disease that has been reported in a few dogs and cats (see Plate 4.*G*).[122-124] Platelet concentrations have been markedly increased (1,000,000–5,000,000/µL). Large, pleomorphic, or hypogranular platelets may be present and similar to those seen with some megakaryocytic leukemias (see Plate 4.*P*). Platelet function may be diminished. Increased numbers of mature megakaryocytes are present in the bone marrow, and myeloid hyperplasia and erythroid hypoplasia have been reported. Though not proven in dogs and cats, the human condition involves a clonal proliferation of pluripotent stem cells.[125] Diagnosis is currently by exclusion of other causes of

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Table 5.3. Diseases and conditions that cause thrombocytosis

Table 5.3. Diseases and conditions that cause thrombocytosis
Hemic neoplasia (clonal thrombocytosis)
Primary (essential) thrombocythemia and other myeloproliferative diseases
Acute megakaryocytic leukemia (M7)
Reactive thrombocytosis (secondary, nonclonal)
Increased production
[a]Inflammation: infection, immune-mediated, surgery, trauma
Nonhemic neoplasia
[a]Iron deficiency
Vinca alkaloids (vincristine, vinblastine)
[a]Recovery from thrombocytopenia (rebound): withdrawal of myelosuppression, recovery from IMT
Splenectomy (post)
Blood loss
Redistribution
Physiologic: exercise, epinephrine
Splenectomy (post)

persistent thrombocytosis. Other myeloproliferative diseases (polycythemia vera, chronic myelogenous leukemia, and myelofibrosis) and myelodysplastic disorders have been associated with thrombocytosis in human patients.

b. Acute megakaryocytic leukemia (M7 subtype of acute myeloid leukemia) has been reported rarely in dogs and cats (see Plate 4.*N*, *O*, and *P*). In contrast to essential thrombocythemia, > 30% of nucleated cells in the marrow are megakaryoblasts, marrow fibrosis may be present, and neoplastic megakaryoblasts may be present in blood and other organs.[69,70,126-128] Thrombocytosis or thrombocytopenia may be present.

2. Reactive thrombocytosis (secondary, nonclonal): Reactive thrombocytosis occurs secondary to other conditions and does not involve neoplasia of the megakaryocyte cell line. In most cases, thrombocytosis is mild to moderate and poses no threat to the patient. It is a nonspecific indicator of certain underlying abnormalities.
   a. Increased production
      (1) Inflammation: Inflammatory cytokines including IL-6 appear to be important mediators of increased thrombopoiesis in a wide variety of infectious and noninfectious inflammatory conditions including surgical and nonsurgical trauma in people.[129,130] Thrombocytosis is frequently associated with inflammatory conditions in horses, dogs, and cats.[131,132]
      (2) Nonhemic malignant neoplasia: Thrombocytosis may result from accompanying inflammation or from production of thrombopoietic cytokines (e.g., IL-6) by neoplastic cells.[133-135]
      (3) Iron deficiency: Thrombocytosis is a common but inconsistent finding in canine and human patients with iron deficiency. The specific cause is not known, but blood concentrations of measured thrombopoietic cytokines (e.g., thrombopoietin, IL-6) have not been increased in human patients with iron deficiency and thrombocytosis.[136]
      (4) *Vinca* alkaloids: Vincristine and vinblastine stimulate thrombopoiesis that can lead to thrombocytosis without increased MPV.[137,138] In patients with IMT,

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these drugs may lead to increased platelet concentrations by other mechanisms, including inhibition of the MPS and therefore decreased platelet destruction.

(5) Recovery from thrombocytopenia (rebound thrombocytosis): Thrombocytopenia may stimulate enough thrombopoiesis that production exceeds consumption/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.[100,139-141]

(6) Postsplenectomy: Splenectomy results in thrombocytosis, sometimes marked, associated with increased thrombopoiesis and increased blood concentrations of thrombopoietin.[142-144] Thrombocytosis is transient, but may persist for weeks. Other mechanisms that may contribute to the thrombocytosis include decreased platelet destruction or decreased sequestration.[145]

(7) Blood loss, especially chronic, has been associated with thrombocytosis in several species, but iron deficiency, inflammation, or neoplasia may cause or contribute to thrombocytosis in many cases. Thrombocytosis occurred with repeated phlebotomy in nonsplenectomized rabbits that were supplemented with iron, but did not occur in iron-supplemented, splenectomized rabbits.[146]

(8) Hypercortisolemia: Hyperadrenocorticism and exogenous glucocorticoids have been associated with thrombocytosis in dogs,[131] but a cause-and-effect relationship is not clear. Thrombocytosis may relate to underlying or concurrent conditions. Prednisone administration to healthy dogs resulted in no increase[137] or a questionable increase[147] in platelet concentrations.

b. Redistribution: Mild and transient physiologic thrombocytosis, even when corrected for hemoconcentration, may occur in some species (e.g., dogs, cats, people) with strenuous exercise or epinephrine release.[12,148-151] In people, the thrombocytosis appears to result primarily from release of platelets from the spleen, but exercise may induce thrombocytosis in asplenic patients.[152] There is evidence for platelet redistribution from lungs.[152,153]

VI. Platelet volume

A. Among individuals, populations, and species, platelet volume is generally inversely related to the platelet concentration.[21]

B. The MPV of a blood sample is the average apparent volume of all the particles in the sample that are counted as platelets. Because MPV is an average, populations of large or small platelets may be present and detectable by microscopy when the MPV is WRI.

1. Analytical concepts
   a. Units: fL
   b. Sample: An accurate MPV requires that all platelets be assessed, that they are not clumped, that their shape has not been altered by activation and pseudopod formation, and that few other particulates such as lipid droplets or cell fragments are detected as platelets.
   c. MPV is commonly measured and often reported as part of a complete blood count using EDTA-anticoagulated whole blood. However, such values are often inaccurate due to methods of measurement and effects of time, temperature, and anticoagulant.

(1) MPV results vary with the type of analyzer.[154] Electrical impedance methods

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may exclude large platelets from evaluation, and the diluting fluids used to sphere platelets in optical analyzers may falsely decrease the values.[155-157]

(2) With impedance measurements, EDTA induces artifactual and time-dependent increases in MPV that occur within 5 min and may result in 3-hr values that are 150% of baseline for room temperature samples.[155,158] Artifactual increases also occur when EDTA samples are refrigerated (4°C).[159] Citrated blood samples maintained at 37°C are preferred but impractical; MPV increases over time in citrated samples stored at room temperature or 4°C, but the increase is less than with EDTA samples.[155]

2. Interpretation of MPV values is limited by inaccuracies and inconsistencies of routine MPV measurement and limited knowledge of factors influencing platelet size,[155,160] especially in domestic species. In general, there is an inverse but nonlinear correlation between platelet concentration and MPV in people without primary bone marrow disorders; MPV values tend to be greater with thrombocytopenia and lower with thrombocytosis.[161] A similar relationship was found in healthy cats.[162] Complete interpretations of MPV require consideration of this relationship.
3. Increased MPV
   a. Increased MPV, when accurate, usually suggests accelerated thrombopoiesis, i.e., an increased stimulus for platelet production.[155] Though there are reports to suggest postproduction size modification of platelets,[141] platelet size is generally thought to be established primarily during production and not dramatically altered in the circulation.[155,163] Therefore, increased MPV may reflect accelerated thrombopoiesis occurring any time in the 5–10 days (platelet lifespan) preceding testing. Increased MPV may precede increases in platelet concentration in patients recovering from thrombocytopenia.[155] However, a strong thrombopoietic stimulus and increased MPV could occur with either adequate or inadequate megakaryocytopoiesis.
   b. Increased production of abnormal platelets in essential thrombocythemia and other clonal disorders may lead to an increased MPV or an unexpectedly high MPV for the degree of thrombocytosis.[154,155]
   c. Abnormally large platelets are produced in certain congenital platelet disorders, so MPV may be increased. Large platelets occurred in the originally described dogs with otterhound thrombopathia,[164] and they are present in many Cavalier King Charles spaniels (see Plate 4.*J*).[165,166]
   d. MPV may increase with physiologic thrombocytosis, possibly due to mobilization of the splenic platelet pool, which is thought to be overrepresented by large platelets in some species.[155]
   e. Increased MPV is associated with hyperthyroxinemia in people and mice.[155]
   f. Acute infection with the Kawakami-Theilen strain of feline leukemia virus induced production of macrothrombocytes.[167] This was associated with decrements in platelet concentration but no significant change in platelet mass.
4. Decreased MPV
   a. Dogs with IMT may have decreased MPV values more often than dogs with thrombocytopenia for other reasons, but values may also be increased or WRI.[168]
   b. With severe thrombocytopenia (< 5000/μL), MPV may be affected by nonplatelet debris in the sample or instrument if special precautions are not taken. This debris is insignificant except at very low platelet concentrations.
   c. Bone marrow failure and chemotherapeutic myelosuppression have been associat-

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ed with decreased MPV in people.[154] However, MPV was WRI for seven of nine dogs with primary bone marrow disease, and increased in two of nine; the latter two dogs did not have megakaryocytic hypoplasia.[168]

C. PDW is a unitless index of platelet anisocytosis calculated by some blood analyzers. An increased PDW indicates an increased population of large platelets, an increased population of small platelets, or both.

D. Platelet volume is also subjectively assessed by microscopic inspection of blood films. This two-dimensional assessment of flattened platelet size is not standardized, but populations of large platelets can be detected even when automated analyzers miss them. Platelets larger than erythrocytes are commonly considered giant platelets, shift platelets, or megathrombocytes (see Plate 4.*K*), though definitions vary. They may appear activated (see Plate 4.*J*), elongated (see Plate 4.*L*), or abnormal (see Plate 4.*P*). An increased population of giant platelets may be present even when the overall MPV is WRI. Interpretation is similar to that of MPV, though there is no standardized, objective method of reporting giant platelets. They can be found in blood samples from healthy animals, especially cats.

VII. Platelet function tests

A. BMBT is a standardized *in vivo* test of primary hemostasis used in dogs and cats. Other mucosal membranes have been used in large animals. The skin bleeding time is less repeatable,[169] and cuticle bleeding times assess secondary hemostasis in addition to primary 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, 3.37 min). Interobserver and intraobserver imprecision[170] suggests it would be most appropriate to report to the nearest minute. The BMBT of healthy dogs has been reported to range from 1 to 5 min, but it usually is less than 4 min.[169,170]

b. Procedure: The upper lip is rolled out and usually secured with a gauze strip around the maxilla. Timing begins when a standardized cut is made in the mucosal surface of the upper lip using a spring-loaded device (Simplate-II, Organon Teknika Corp. or Surgicutt, International Technidyne). The cut is small enough (5 mm × 1 mm) for primary hemostasis alone to resolve the bleeding; coagulation and generation of fibrin is not necessary. Filter paper is used to blot away excess blood without touching or disturbing the incision itself. The endpoint is when bleeding ceases and a crescent of blood no longer forms on the filter paper.

2. The BMBT test is relatively insensitive, but BMBT will be prolonged (greater than about 4-5 min in dogs) with moderate to marked defects of primary hemostasis.

a. Thrombocytopenia (Table 5.2): Marked thrombocytopenia is a contraindication to BMBT because it is already known that BMBT will be prolonged. The degree of thrombocytopenia required to prolong the BMBT is not clear, but it is commonly stated that BMBT prolongations may occur with platelet concentrations at or below 100,000/µL. This cutoff probably varies with other factors, such as MPV, vWf:Ag, or Hct.

b. Thrombopathia (platelet dysfunction) (Table 5.4)

(1) Terms used to describe a disorder of abnormal platelet function include *thrombopathia, thrombopathy, thrombocytopathy*, and *thrombocytopathia*. The

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Table 5.4. Diseases and conditions that cause decreased platelet function

Hereditary: Basset hound thrombopathia, Chédiak Higashi syndrome (Persian cats; Hereford, Brangus, and Japanese black cattle), cyclic hematopoiesis (grey collie), dense granule storage pool disease of American cocker spaniels, Glanzmann's thrombasthenia (great Pyrenees, otterhounds), Simmental hereditary thrombopathia, Spitz thrombopathia, thrombasthenic thrombopathia (otterhounds–original variant)

Acquired

- [a]Drugs: anesthetics (barbiturates), anti-inflammatories (NSAIDs), membrane-active drugs (local anesthetics, beta blockers, antihistamines), antibiotics (penicillins and cephalosporins), antiplatelets (ticlopidine), dietary factors (garlic, ethanol, caffeine)
- Envenomation (certain venoms)
- [a]FDPs (increased fibrinolysis, DIC)
- Hepatic disease
- Hyperglobulinemia (multiple myeloma, ehrlichiosis)
- IMT (some antiplatelet antibodies)
- Infections: BVD, FeLV
- Neoplasia involving megakaryocytes: myeloproliferative diseases, acute megakaryocytic leukemia
- [a]Renal failure (uremia)
- Synthetic colloids (dextran, hetastarch)

Note: Decreased platelet function should be considered when (1) petechiae, ecchymoses, or mucosal hemorrhages occur without marked thrombocytopenia or (2) bleeding time or clot retraction are prolonged in the absence of thrombocytopenia (also consider vWD). Thrombopathia can be documented by abnormal platelet aggregometry, adhesion, or secretion studies.

precise definition and usage of each term varies. *Thrombopathia* will be used herein as a general term to indicate any disorder of platelet function.

(2) Thrombopathia should be suspected if BMBT is prolonged but platelet concentrations, Hct, and vWf:Ag values are WRI. Hereditary thrombopathias are uncommon; additional reading is available.[164] Acquired thrombopathia occurs concurrently with diseases or conditions that are usually diagnosed by other findings. The thrombopathia may be subclinical or it may contribute obviously to morbidity.

c. vWD: BMBT can be used as a screening test for vWD in breeds predisposed to the disease (e.g., Doberman pinschers). BMBT should be prolonged when vWf:Ag < 20% and may be prolonged with greater values.

d. Anemia: Anemia prolongs BMBT, possibly by decreasing the interaction of platelets with the vascular wall or because of decreased erythrocyte ADP, a platelet agonist.[171,172]

e. Vascular disease: BMBT rarely may be prolonged with certain vascular diseases.

f. Antiplatelet drugs: Aspirin led to an increase in canine BMBT values but values were still WRI. Other antiplatelet drugs often do not prolong the BMBT.[173,174]

3. Defects restricted to secondary hemostasis and the formation of fibrin (e.g., hemophilia) should not prolong BMBT unless a larger vessel is cut.[175] However, some human patients with afibrinogenemia have prolonged bleeding times, presumably due to the lack of fibrinogen for interplatelet bridging.[176,177]

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4. Bleeding time has not been useful as a screening test for predicting the occurrence of excessive surgical bleeding in human patients.[174]

B. Clot retraction is mediated by platelets, so some thrombopathias (Table 5.4) or marked thrombocytopenia (Table 5.2) can prolong clot retraction. This may be noted by a decreased yield of serum after 60–90 min of clotting. Standardized tests to quantify clot retraction have been developed but are not routinely done.[137,178,179]

C. Other specialized tests are used to assess platelet adhesion, aggregation, and secretion *in vitro*.[164] These tests are available in labs with specialized equipment (aggregometers, PFA-100 analyzer [Dade Behring, Inc., Miami, Florida]) and expertise. In addition to detecting and characterizing decreased platelet function, these tests can also detect hyperactive platelets as may occur with infections (e.g., FIP, heartworm disease), malignancies, the nephrotic syndrome, and other disorders.[180-183]

VIII. Reticulated platelets

A. Just as the RNA content of young erythrocytes (reticulocytes) is used to assess erythropoiesis, "reticulated platelets" have been measured to assess thrombopoiesis in dogs and horses.[184] Reticulated platelets have been shown to be less than 24 hr old in dogs.[185]

B. The assay is primarily a research tool at this time. Platelets are incubated with thiazole orange, which binds to platelet RNA and granule nucleotides and emits a fluorescence that can be detected by flow cytometry. The percentage of platelets with increased fluorescence is determined, and this value can be used in conjunction with the platelet concentration to calculate the concentration of reticulated platelets in the blood. An increased concentration of reticulated platelets is evidence for increased thrombopoiesis. However, the increase in fluorescence may relate either to an increase in platelet size or to an increase in RNA concentration within the platelets, depending upon the method used and how the threshold limit for increased fluorescence is defined.[186-188]

C. Increased concentrations of reticulated platelets may not be present in patients with accelerated thrombopoiesis if the mean platelet lifespan is reduced to less than the period of time a reticulated platelet is detectable.[8,189]

D. Erythropoietin administration to dogs was associated with increased numbers of reticulated platelets and platelet hyperreactivity.[190]

E. This procedure may become clinically useful once it is better standardized and the determinants of platelet size are better understood.

IX. Platelet surface-associated immunoglobulin (PSAIg) and related tests for immune-mediated thrombocytopenia (IMT)

A. A variety of specialized assays have been developed to aid in the diagnosis of canine IMT, but the diagnosis remains primarily one of exclusion. No veterinary assay has undergone rigorous and extensive controlled clinical testing.

B. Sample conditions (e.g., anticoagulant, storage time, storage temperature) may affect results. For example, a few hours exposure of citrated blood samples to room temperature or refrigeration led to variable but sometimes marked increases in PSAIg in canine samples.[191] Storage-related increases in PSAIg also occur with EDTA anticoagulation,[192] but the relative contribution of anticoagulant, storage temperature, and other factors to these increases is not known.

C. Platelet assays

1. Direct platelet assays that test for antibodies on the surface of a patient's platelets (PSAIg) are recommended. Most current assays are flow cytometric; PSAIg is detect-

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ed by fluorescence-tagged, species-specific, anti-immunoglobulin antibodies.[87,88,193-196] Positive results generated from properly processed samples using reliable assays support antibody-mediated platelet destruction but do not differentiate primary IMT from secondary IMT. Negative results suggest the need to search for a nonimmune pathogenesis for the thrombocytopenia.

2. Indirect assays testing for antibodies in a patient's plasma or serum that can bind to "normal" platelets from healthy animals have been used but are not recommended. They are less sensitive and less specific for IMT than are direct assays. Indirect assays do not differentiate autoantibodies from immune complexes, immunoglobulin aggregates that can form in frozen sera, or acquired and naturally occurring alloantibodies to platelet antigens.

D. 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.[94,197] 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, results may be positive.[198] False positive results may occur if megakaryocytes are damaged and cytoplasmic immunoglobulin rather than surface immunoglobulin is detected. The cumulative reported diagnostic sensitivity for clinical diagnoses of canine IMT is about 50%, too low to recommend.[79]

VON WILLEBRAND FACTOR (vWf)

I. Physiologic processes

A. vWf is a large multimeric plasma glycoprotein ($M_r \approx 500{,}000–20{,}000{,}000$) that bridges platelets to injured vessel walls via platelet GPIb and exposed subendothelial proteins such as collagen (adhesion). It also contributes to platelet-platelet bridging via GPIb and the platelet integrin $\alpha_{IIb}\beta_3$ (aggregation). The largest multimers of vWf are the most functional.[199]

B. Most vWf is produced by endothelial cells[200,201] and secreted constitutively or stored and secreted later upon endothelial cell activation. Megakaryocytes and platelets contain a significant amount of vWf in some species (e.g., cats, human beings) but very little in dogs.[199,202,203] Secreted vWf forms noncovalent complexes with coagulation Factor VIII and serves as a stabilizing and protective carrier molecule for it.[204]

II. von Willebrand disease (vWD)

A. vWD is a disorder of primary hemostasis caused by a deficiency of functional vWf. It is the most common hereditary bleeding disorder in dogs. It is rare in cattle,[205] cats,[206] and horses.[207]

B. vWD is usually an inherited disorder, but acquired vWD occurs rarely in people.[208,209] Acquired vWD has not been clearly documented in veterinary species.

1. One of the diseases associated with human acquired vWD is hypothyroidism, and treatment of these people for hypothyroidism has resolved the concurrent vWf deficiencies.[210]

2. An association between hypothyroidism and vWD has also been suggested in dogs, particularly in Doberman pinschers.[211] However, studies have produced conflicting results, and the concurrence of common diseases does not prove a cause-and-effect relationship.[199] The finding of vWf:Ag values that were WRI for hypothyroid dogs before treatment with levothyroxine and significantly decreased (not increased) after

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treatment indicates that there is not a predictable association between hypothyroidism and vWD in dogs.[212]

C. Three general types of vWD have been defined.[213]
   1. Type 1: all vWf multimers are present but at decreased concentrations; variable severity; most common form; occurs in many dog breeds, including Doberman pinschers[214]
   2. Type 2: deficiency of vWf with disproportionate decrease in large multimers; severe form; uncommon; occurs in German shorthaired pointers and German wirehaired pointers;[215] reported in one horse[207]
   3. Type 3: absence of all vWf multimers; severe form; occurs particularly in Chesapeake Bay retrievers, Dutch kooikers, Scottish terriers, and Shetland sheepdogs[216-219]

D. Clinical and laboratory signs of vWD
   1. Mild to severe mucosal hemorrhage (epistaxis, gastrointestinal hemorrhage, prolonged estral bleeding), cutaneous bruising, and prolonged hemorrhage from nonsurgical or surgical trauma (e.g., tail docking, dewclaw removal in puppies, tooth extraction)
   2. Absence of petechiae may help differentiate vWD from platelet disorders.
   3. Prolonged BMBT without thrombocytopenia and without prolonged coagulation times
   4. PTT may be mildly prolonged because decreased Factor VIII coagulation activity (which may be denoted FVIII:C) occurs secondary to reductions in circulating vWf, a carrier molecule for Factor VIII. However, in contrast to human patients with vWD, FVIII:C in dogs with vWD is usually greater than 30% of the activity in reference plasma, so PTT is usually WRI, even in dogs with type 3 vWD and therefore no vWf.[199,220]

III. Analytical concepts

A. Sample
   1. Plasma from blood drawn into sodium citrate or EDTA; citrate tubes should be filled to give a 1:9 volume ratio of citrate to blood. vWf:Ag may be markedly decreased in samples with clots or *in vitro* hemolysis, but lipemia has no significant effect.[221]
   2. vWf:Ag is reportedly stable for at least 8 hr in canine plasma or whole blood stored at room temperature.[221] However, values are significantly increased at 24 hr after collection when whole blood samples are stored at room temperature, and values are increased at 48 hr (but not 24 hr) after collection when plasma is stored at room temperature.[222] These increases did not occur when samples were refrigerated. It is generally recommended that plasma should be collected promptly after blood collection, frozen, and shipped overnight with ice.

B. Units: Percent, 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 samples). Variations in the composition of pooled plasma from the reference animals can affect results.

C. vWf:Ag assays[199,223-225]
   1. ELISA: vWf is usually measured by a quantitative ELISA method with species-specific antibodies to vWf (i.e., vWf:Ag); values of < 50% are usually considered decreased. ELISA testing has largely replaced electroimmunoassay methods involving electrophoresis of plasma vWf in agarose gels containing antibodies to vWf.

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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. Differentiates type 1 vWD (high molecular weight multimers present) from type 2 vWD (high molecular weight multimers absent)
3. Functional assays
   a. Botrocetin cofactor assay: Platelets agglutinate in the presence of botrocetin and vWf. The rate of this vWf-dependent platelet agglutination correlates well with the amount of plasma vWf:Ag except with type 2 vWD. In type 2 vWD, botrocetin cofactor activity may be markedly reduced while the amount of plasma vWf:Ag may be WRI or mildly decreased. This is because the more functional large multimers are deficient.
   b. vWf-collagen binding activity may be measured in canine plasma by an ELISA procedure that detects only the vWf that can bind to collagen. The assay is therefore a measure of vWf quantity and function.

IV. Interpretive considerations
   A. The amount of plasma vWf:Ag has been shown to vary considerably from day to day when healthy or diseased (vWD) dogs are sampled serially.[221]
   B. Amounts of plasma vWf:Ag may be increased after very strenuous exercise;[226,227] vWf:Ag increased as much as 100% within minutes after strenuous exercise in horses.[228] Similar increases occurred in bitches at parturition, with lesser increases in the last trimester of pregnancy and for the first 1-2 weeks after parturition.[229] Values may also be increased by epinephrine,[226] endotoxin,[230] and DDAVP (1-deamino-8-D-arginine vasopressin).[226] DDAVP increases vWf:Ag in dogs with type 1 vWD or in dogs without vWD by releasing vWf, especially the larger multimers, from endothelial cells.[231] DDAVP can be used effectively to increase the amount of vWf in blood donor animals when given 30 to 90 min prior to blood collection.[232] Increases in the amount of plasma vWf:Ag may also occur with azotemia,[233] liver disease,[234] and other illnesses.

V. Decreased von Willebrand factor:antigen (vWf:Ag) (Table 5.5)
   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.[235]
      1. Dogs with vWf:Ag values < 50% 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.

Table 5.5. Diseases and conditions that cause decreased vWf:Ag

vWD: vWf:Ag usually < 35%
[a]Type 1: all vWf multimers present but proportionately deficient
Type 2: deficiency of vWf multimers with high M_r
Type 3: absence of all vWf multimers, vWf:Ag ≈ 0%
[a]vWD carrier (rarely symptomatic): vWf:Ag usually 30%–70%

Notes: Hemolysis or clotted samples may cause marked false decreases in vWf:Ag values. vWf:Ag units: percent of result for plasma pooled from healthy dogs.

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2. Dogs with borderline vWf:Ag values of 50%–69% 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 affected (value < 50%) or not (value > 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 70%–180% plasma vWf:Ag 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. Multimeric analysis is necessary to document type 2 vWD. Functional testing may be suggestive.

VI. Genetic tests: Genetic tests have been developed that can detect some vWf gene mutations in several breeds of dogs. These tests may prove useful to detect carriers in specific breeds.[219,236]

COAGULATION

I. Physiologic processes[237]

A. Blood coagulation is intimately associated with endothelial cells, platelets, the fibrinolytic system, and other blood cells, but it can be evaluated independently.

B. Coagulation involves an interconnected series of enzyme-activating steps resulting in the formation of thrombin (Factor IIa) and the conversion of soluble fibrinogen (Factor I) into an insoluble fibrin plug called the secondary hemostatic plug.

C. Most steps involve an enzyme, a substrate (fibrinogen, fibrin, or proenzyme forms of coagulation enzymes), and a cofactor (e.g., Factors Va and VIIIa) assembled and localized on a phospholipid surface (e.g., platelet, leukocyte, or endothelial cell membranes) in the presence of fCa^{2+}.

D. The coagulation cascade or web can be divided into three pathways that can be assessed separately *in vitro* but which have considerable cross talk *in vivo* (Fig. 5.3).

E. Coagulation factors (Table 5.6)

1. Enzymatic coagulation factors circulate as inactive proenzymes (zymogens) until they are activated.
   a. They are produced primarily in hepatocytes; the production of Factors II, VII, IX, and X is vitamin K-dependent (Fig. 5.4). The "K" in vitamin K is derived from the German *Koagulations vitamin* (i.e., the coagulation vitamin).[237]
   b. Factor IX is sex-linked; its gene is on the X chromosome.[238,239]
   c. Half-lives of proenzymes in health: In humans, half-lives range from a few hours (Factor VII) to several days (Factors II and XIII); most are about 1–2 days. Values are presumed to be similar for domestic mammals.
   d. Most enzymatic factors are not directly destroyed during coagulation; 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., Factor XIII by plasmin) does occur.
2. Nonenzymatic coagulation factors include fibrinogen, protein pro-cofactors (Factors V and VIII), fCa^{2+}, and phospholipid.
   a. Fibrinogen is a positive acute-phase protein produced by hepatocytes. It has a half-life of 2–3 days in healthy dogs,[116] and it is consumed during coagulation by thrombin cleavage to fibrin.

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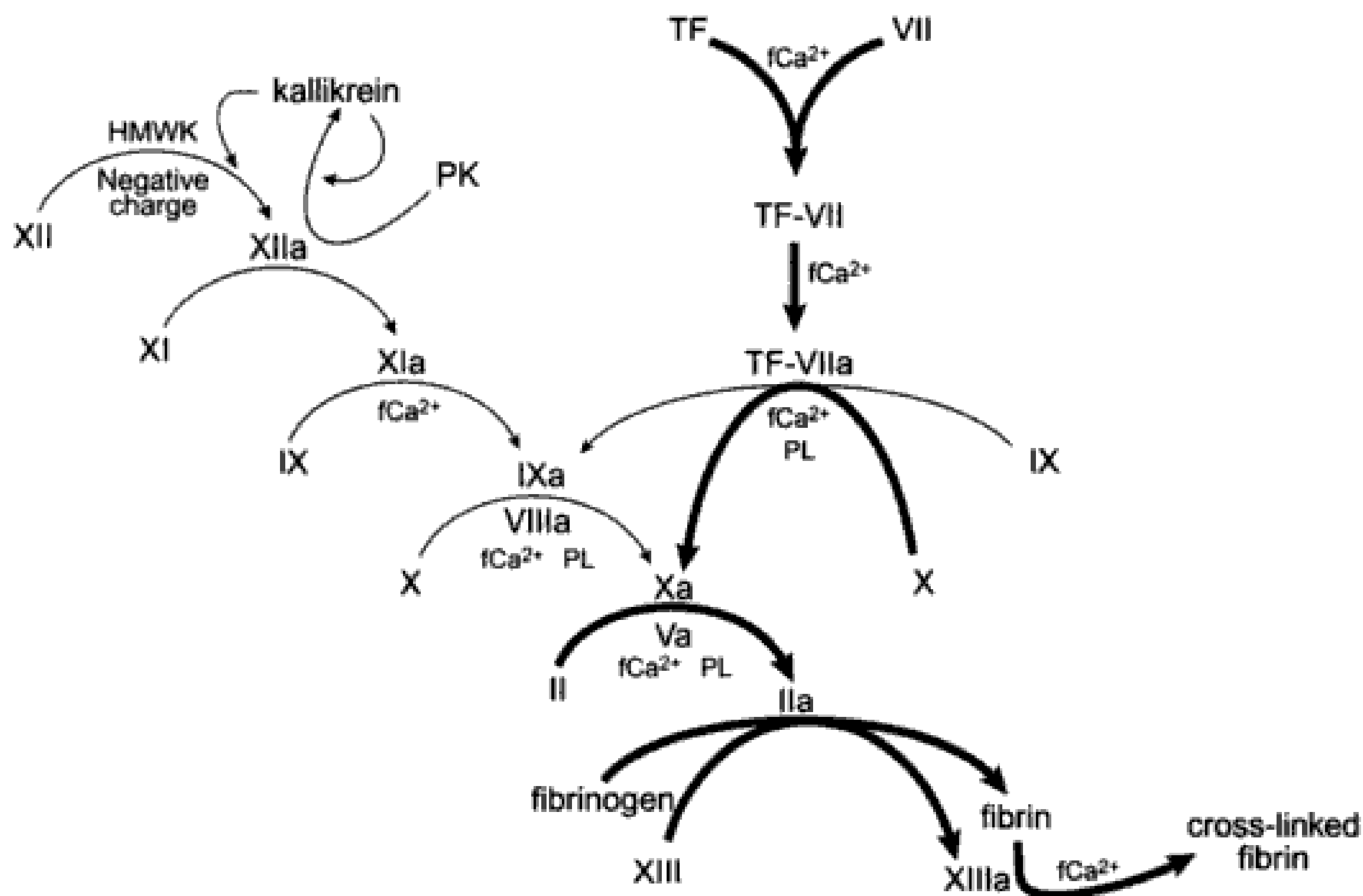

Fig. 5.3. The coagulation cascade begins with activation of the extrinsic (TF) or intrinsic (Factor XII) pathways and results in formation of cross-linked fibrin by the common pathway (beginning with Factor X). Bold arrows represent what is believed to be the major coagulation pathway *in vivo*.

- *Extrinsic pathway:* It was originally thought to require extravascular activation, hence the name extrinsic. This pathway is initiated by TF released from, or exposed on, damaged tissue or activated endothelial cells, monocytes, or macrophages. These cells can be activated by endotoxin and certain inflammatory cytokines. TF binds to Factor VII in the presence of fCa^{2+} and is rapidly activated to Factor VIIa in a TF-VIIa complex. TF-VIIa rapidly activates Factor X (common pathway) and Factor IX (intrinsic pathway) in the presence of fCa^{2+} and phospholipid (PL).
- *Intrinsic pathway:* It was originally considered the pathway activated by intravascular (intrinsic) factors. This pathway is initiated by so-called contact activation, the activation of Factor XII by contact with a negatively charged surface. *In vivo*, this could be subendothelial collagen exposed at the site of vascular injury. *In vitro*, kaolin, diatomaceous earth, or glass surfaces may be involved.
  - Once formed, surface-bound Factor XIIa facilitates the binding of HMWK to the activating surface, probably by enzymatic cleavage of HMWK. Because HMWK circulates in association with PK and Factor XI, all four contact activation factors (PK, HMWK, Factors XI and XIIa) become closely associated.
  - Factor XIIa activates PK to kallikrein, which enzymatically produces more kallikrein and more Factor XIIa in a potent amplification pathway.
  - Factor XIIa cleaves Factor XI, yielding Factor XIa, which cleaves Factor IX in the presence of fCa^{2+} to form Factor IXa. Factor IXa then binds to the PL surface (in the presence of fCa^{2+}), and there, in association with Factor VIIIa (activated mostly by thrombin), it cleaves Factor X to form Factor Xa.
- *Common pathway:* This is the common end to the extrinsic and intrinsic pathways, beginning with the activation of Factor X. Factor Xa complexes with Factor Va (activated mostly by thrombin) and fCa^{2+} on a phospholipid surface to form the active prothrombinase complex, which results in the enzymatic conversion of prothrombin (Factor II) to thrombin (Factor IIa). Thrombin then cleaves its many substrates (not all shown), which include:
  - Fibrinogen: Fibrinopeptides A and B are cleaved from fibrinogen to form fibrin monomers, which polymerize into fibrin polymers.
  - Factor XIII: Proteolytic cleavage of Factor XIII leads to activation; Factor XIIIa, in the presence of fCa^{2+}, cross-links fibrin and reinforces the secondary hemostatic plug.
  - Protein pro-cofactors (Factors V and Factor VIII): Proteolytic cleavage leads to cofactor activation and accelerated coagulation.
  - Factor XI: Thrombin activation provides positive feedback on the intrinsic and common pathways through Factor XIa.
  - Protein C: Forms activated protein C which inactivates Factors Va and VIIIa, and promotes fibrinolysis via t-PA.

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Table 5.6. Coagulation factors, abbreviations, and roles

Factor	Name	Pathway	Function
I	Fibrinogen	Common	Substrate for thrombin–converted to fibrin
II	Prothrombin	Common	Proenzyme: IIa (thrombin) cleaves fibrinogen and activates V, VIII, XI, XIII, protein C, and platelets
III	Tissue factor (TF)	Extrinsic	Cofactor: TF binds and activates VII, and the TF-VIIa complex activates IX and X
IV	Ionized calcium (free)	All	Cofactor for IIa, VIIa, IXa, Xa, and XIIIa
V	Proaccelerin	Common	Pro-cofactor for Xa
VII	Proconvertin, stable factor	Extrinsic	Proenzyme: VIIa activates IX and X
VIII	Antihemophilic factor	Intrinsic	Pro-cofactor for IXa
IX	Christmas factor	Intrinsic	Proenzyme: IXa activates X
X	Stuart factor, Stuart-Prower factor	Common	Proenzyme: Xa activates II
XI	Plasma thrombo-plastin antecedent	Intrinsic	Proenzyme: XIa activates IX
XII	Hageman factor	Intrinsic	Proenzyme: XIIa activates XI, PK, and HMWK
XIII	Fibrin stabilizing factor, fibrinase	Common	Proenzyme: XIIIa cross-links fibrin and protects it from plasmin degradation
HMWK	Fitzgerald factor	Intrinsic	Cofactor for activation of XII and XI
PF3	Platelet factor 3	Intrinsic, common	Negatively charged platelet membrane lipoprotein important for *in vivo* activation of X and II
PK	Fletcher factor	Intrinsic	Proenzyme: kallikrein activates XII and PK, generates bradykinin from HMWK, and leads to plasmin generation

b. Factors V and VIII markedly accelerate coagulation by facilitating surface attachment and localization of coagulation factors.
   (1) Factor V production may occur in hepatocytes, megakaryocytes, lymphocytes, and vascular smooth muscle cells.[240] Its half-life is about 0.5–1.5 days, and it is consumed by protein Ca (activated protein C) during coagulation.
   (2) Factor VIII production may occur in multiple cell types, but hepatocytes appear to be most important. It is sex-linked; its gene is on the X chromosome.[241] It circulates in a noncovalent complex with vWf, but it is distinct from vWf. The half-life of human Factor VIII is about 0.5 days, but it is less in the absence of vWf, its carrier protein. It is consumed by protein Ca during coagulation, and it is a positive acute-phase protein.[242,243]

c. EDTA, oxalate, and citrate function as anticoagulants by binding fCa^{2+} and preventing it from interacting with coagulation proteins.

F. Physiologic inhibitors of coagulation help prevent excessive coagulation; deficiencies are associated with thromboembolic disease. Some of the inhibitors can be measured.
   1. ATIII is the major inhibitor of coagulation enzymes. It is a protein ($M_r \approx 58{,}000$) produced primarily by hepatocytes. It binds, inactivates, and removes most coagula-

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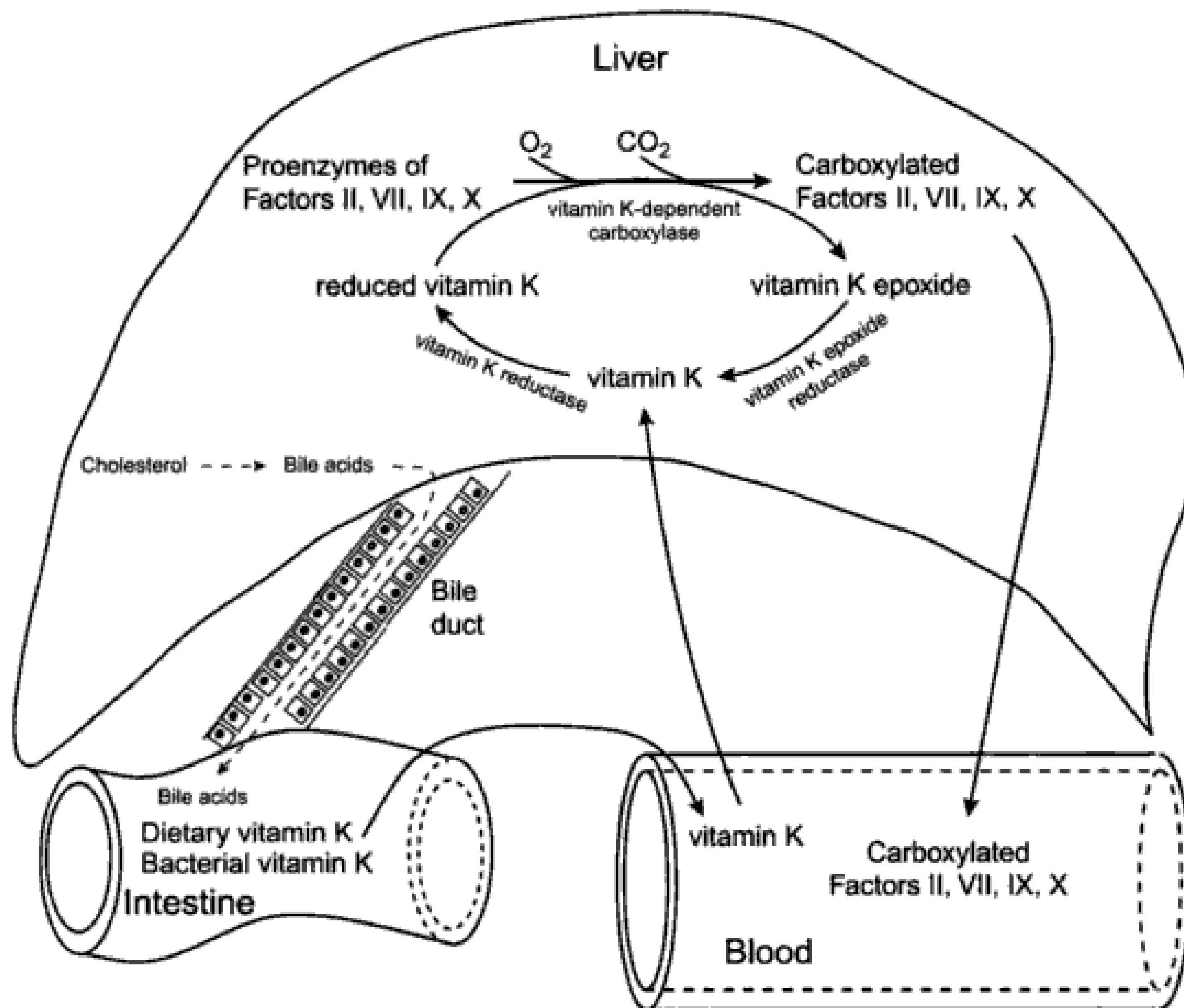

Fig. 5.4. Coagulation Factors II (prothrombin), VII, IX, and X are synthesized primarily in hepatocytes. Vitamin K is required for these factors to be functional, i.e., they are vitamin K dependent.

- 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 the action of bile acids.
- In hepatocytes, vitamin K becomes reduced to its active form (reduced vitamin K). Reduced vitamin K is a cofactor for vitamin K-dependent carboxylase, the enzyme responsible for posttranslational gamma-carboxylation of glutamic acid residues in these coagulation factors. Carboxylation is needed so that the factors can bind fCa^{2+}, which induces conformational changes and allows binding to phospholipid membranes.
- 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.
- Carboxylated Factors II, VII, IX, and X enter the blood, where they can be activated to participate in enzymatic reactions of the coagulation system.

tion enzymes—most importantly thrombin, Factor IXa, and Factor Xa—from circulation via receptors on hepatocytes.[244] ATIII-enzyme complexes are rapidly cleared by hepatocytes. ATIII activity is markedly enhanced by heparin (exogenous, or endogenous from mast cells) and heparan sulfate on endothelial cells (Fig. 5.5).

2. Protein C (the "C" stands for the third [a, b, c] fraction eluted from a column)[245] is a vitamin K–dependent proenzyme anticoagulant ($M_r \approx 62{,}000$) produced in hepatocytes. It circulates in plasma with a half-life of 8-10 hr in people. It can be activated by thrombin, primarily when thrombin is bound to thrombomodulin, a protein thrombin receptor present on most endothelial cell membranes. Protein Ca then inactivates Factors Va and VIIIa by proteolytic cleavage in the presence of Factor V[246] and membrane-bound protein S (the "S" stands for Seattle, where the protein was

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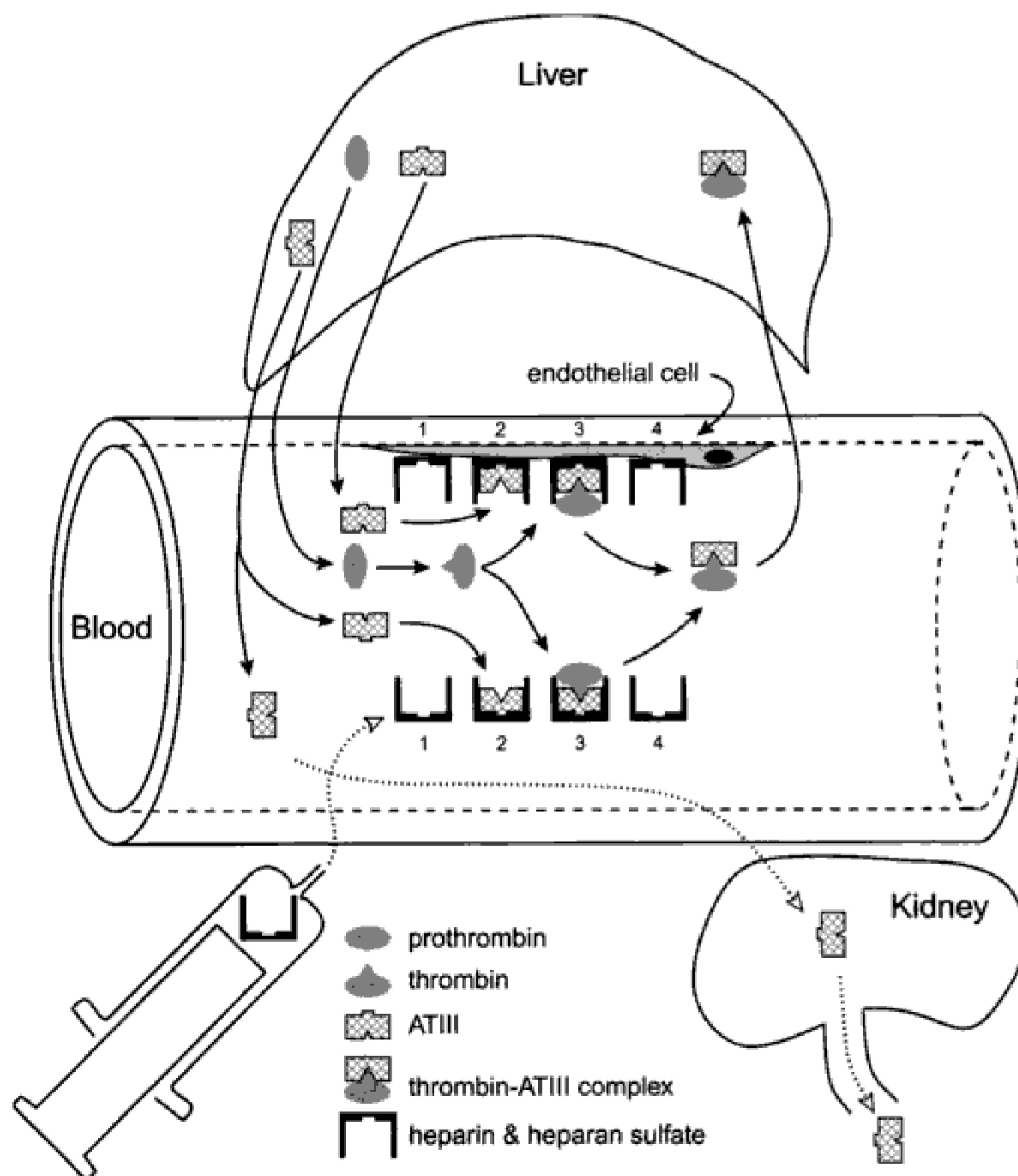

Fig. 5.5. ATIII is the major physiologic anticoagulant in blood. It is produced primarily by hepatocytes, and it circulates in the blood, where it can inactivate thrombin (shown here) and the other coagulation enzymes. As shown schematically, ATIII activity is markedly enhanced in the presence of circulating heparin or heparan sulfate on endothelial cells (1). Heparin or heparan sulfate bind to lysine sites of ATIII (2), inducing a conformational change in ATIII (note larger triangular notches) that increases its affinity for thrombin. Thrombin, generated from the activation of prothrombin, then binds to the heparin-ATIII complex, forming a covalent 1:1 complex with ATIII (3). The thrombin-ATIII (TAT) complex dissociates from the heparin or heparan sulfate and is cleared from circulation by hepatocytes. The heparin and heparan sulfate act as catalysts and are available (4) for forming more complexes. Anticoagulation by ATIII-heparin limits excessive clotting, but ATIII-heparin does not inhibit coagulation enzymes bound to fibrin or platelets. Therefore, localized and controlled coagulation can proceed where needed.

- Decreased plasma [ATIII] occurs via consumption when there is increased intravascular coagulation or after injection of exogenous heparin. (Nonphysiologic pathways are represented by dotted arrows.) Decreased concentrations may also occur from decreased hepatic production or from excessive renal loss due to protein-losing nephropathy. Decreased ATIII produces a prothrombotic state that heparin cannot resolve because heparin requires ATIII for most of its function.
- Increased plasma [ATIII] may occur with increased hepatic production of ATIII. This may occur secondary to the production of inflammatory cytokines.

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first described).[245] Protein S is a vitamin K–dependent, nonenzymatic cofactor produced by endothelial cells, hepatocytes, and megakaryocytes. Additional anticoagulation occurs by this pathway because thrombin bound to thrombomodulin cannot cleave fibrinogen, and the complex is internalized and degraded by endothelial cells. Also, protein Ca promotes fibrinolysis.

3. TFPI ($M_r \approx 40{,}000$) circulates in platelets and in plasma, mostly bound to lipoproteins. In the presence of fCa^{2+}, TFPI inhibits TF-VIIa by forming a stable quaternary complex: TF-VIIa-Factor Xa-TFPI.[247] This inhibits further generation of Factor Xa via TF-VIIa.
4. Other circulating inhibitors of coagulation include heparin cofactor II, α_2-macroglobulin, and α_1-proteinase.

II. Analytical concepts

A. Assay optimization for one species (e.g., human) does not necessarily optimize for other species.[248-251] Veterinary assays for routine coagulation testing are not standardized and many are not optimized for the species being tested. This compromises their sensitivity to detect impaired coagulation.

B. Proper sampling and sample handling are critical for accurate results from most coagulation assays.

C. Sample collection

1. Use a citrate vacuum tube or plastic syringe containing the right amount of citrate for the volume of blood to be drawn. Sampling through nonheparinized catheters may be done, if necessary, after flushing the catheter with 5 mL saline and discarding at least the first 5 mL of blood removed (at least 6 times the catheter dead space).[252,253] Sampling through heparinized catheters should be avoided, though a similar flush and discard approach produced results for *healthy* dogs that did not differ significantly from direct venipuncture results.[254]
2. Care should be taken to minimize activation of platelets and the coagulation and fibrinolytic systems. Traumatic venipuncture exposes blood to TF, thus initiating coagulation that can result in clotted samples or platelet activation and clumping. Freely flowing blood should be collected by "clean" venipuncture on the first attempt. Probing with the needle, sampling through a hematoma, or exiting and reentering the vein will expose the blood to TF and activate the pathways to be tested. If collection is difficult, a new vein should be selected. Excessive vacuum may cause turbulence and platelet activation. The first few drops or a complete tube may be discarded to decrease TF in the test sample. The blood and anticoagulant should be mixed immediately and thoroughly, but gently.

D. Sample

1. Most coagulation tests: citrated plasma
2. Point-of-care instruments: citrated whole blood or citrated plasma
3. ACT: whole blood in special ACT tubes containing diatomaceous earth
4. Lee-White WBCT: uncoagulated whole blood tested immediately
5. For citrated samples, plasma or whole blood should be anticoagulated with trisodium citrate at a 1:9 (anticoagulant:blood) ratio using 3.2% or 3.8% citrate tubes (check with laboratory for preference). The citrate concentration significantly affects the results of coagulation tests on human samples (3.2% is usually recommended),[253,255,256] and it can have significant effects on some results with canine samples.[257] The magnitude of the effect is not known for most conditions or most species, but a

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standard citrate concentration with reference intervals generated using that citrate concentration, instrument, and method are recommended.

6. Similarly, it is important to maintain the 1:9 citrate:blood volume ratio because overcitrated samples (too little blood) may have reduced coagulation activity (prolonged times) and undercitrated samples (too much blood) may 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.[258]
7. Even with the correct blood volume, Hct may affect the appropriate amount of citrate to use. With anemia, plasma may be undercitrated because there is more plasma with the same amount of citrate (reduced coagulation times); with erythrocytosis, plasma may be overcitrated because there is less plasma with the same amount of citrate (prolonged coagulation times).[259]
   a. Decreased citrate:blood ratios are recommended when Hct > 55% in people.[253] Increased ratios may be indicated for Hct < 25%.
   b. To maintain the desired anticoagulant concentration in a 3 mL sample, approximately 50 µL less anticoagulant should be used for each 10 percentage-point increase in Hct above 45%.

E. Sample processing and stability[253]
   1. Except for ACT and Lee-White WBCT assays, samples may be refrigerated or kept at room temperature for up to 4 hr (PTT assay for nonheparinized patients, TT assay, coagulation factor analyses) or up to 24 hr (PT assay) before processing. However, a good general recommendation is to centrifuge and remove plasma within 1 hr (room temperature storage) and test within 4 hr of sample collection, because this time frame is necessary for certain tests and certain samples.[260]
   2. After confirming the absence of clots in the sample, blood should be centrifuged for 10–15 min at high g-force (e.g., 15 min at 1500 × g), and the platelet-poor plasma should be removed by plastic pipette. If the plasma cannot be tested within 4 hr, it should be frozen. Excessive platelets (10,000/µL to 200,000/µL) remaining in the plasma as a result of inadequate centrifugation forces will not interfere with PT and PTT assays for routine diagnostic work but will interfere with heparin monitoring (via PTT assay) and tests for other inhibitors of coagulation.[261,262]
   3. Frozen human plasma may be stored for 2 weeks at -20°C or for 6 months at -70°C before testing, but frost-free freezers should be avoided. Stability studies of common hemostatic analytes in canine plasma suggest a similar stability at -70°C for all plasma tests but PTT.[263] Frozen plasma should be mailed with ice to arrive within 24 hr. Samples should be thawed rapidly to minimize cryoprecipitate formation and therefore loss of hemostatic factors. They should be tested immediately after thawing. PTT may be affected by freezing. 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. 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. Plasma should not be placed in a "serum tube" containing a clot activator; this will promote coagulation.

F. Instruments: A variety of automated coagulation analyzers, including point-of-care instruments, are replacing manual methods of measuring coagulation times for human and veterinary samples. Specific methods and sample requirements vary with the analyzer.

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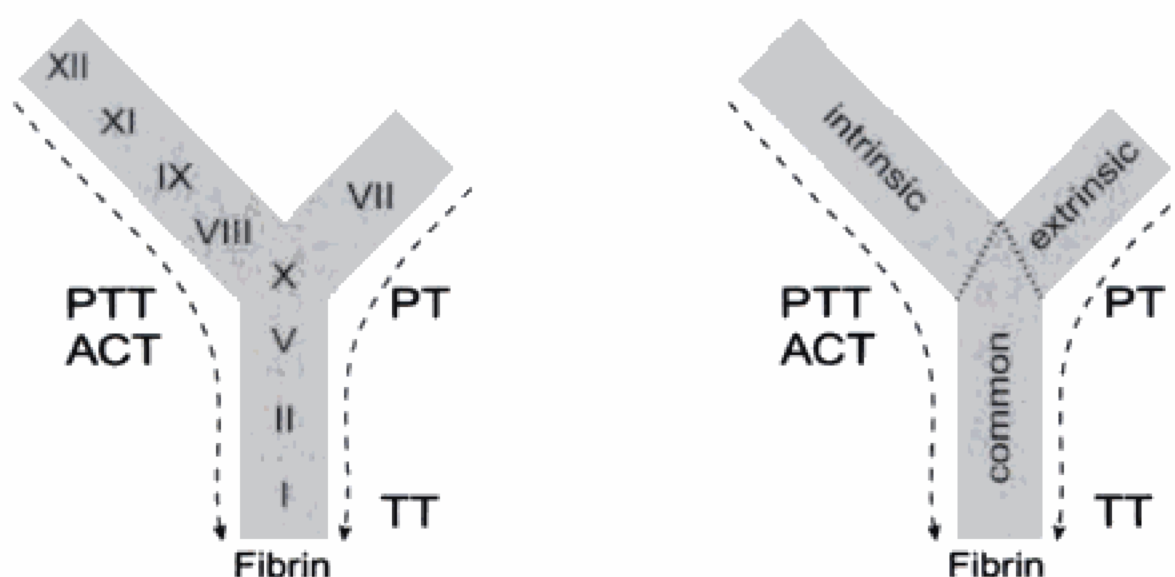

Fig. 5.6. Schematic representation of the coagulation cascade as evaluated by *in vitro* screening coagulation tests.

Fibrin formation is the endpoint for each test. PTT and ACT evaluate coagulation factors in the intrinsic arm (Factors XII, XI, IX, and VIII) and common pathway (Factors X, V, II, and I). PT evaluates the short extrinsic arm (Factor VII) and the common pathway. TT assesses only the conversion of fibrinogen (Factor I) to fibrin with the addition of thrombin.

G. General analytical approach: *In vitro* analysis allows independent testing of different parts of the coagulation web, simplified to a "Y", so that defects may be localized within the intrinsic, extrinsic, or common pathways (Fig. 5.6). Testing generally begins with common screening tests; more specialized tests can be used when indicated.

III. Whole blood clotting time (WBCT)

A. Lee-White methods[264,265]

1. A standardized, but rarely used, insensitive method of screening for defects in the *intrinsic* and *common* pathways using nonanticoagulated whole blood immediately after collection; contact activation of coagulation is initiated by the glass tube.
2. 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.[266] Variations in protocol, including venipuncture technique, blood volume, glass tubes size and coating, temperature, and sample Hct, influence the results.
3. Reported reference values are 4–15 min for horses and cattle, 3–13 min for dogs, and about 8 min for cats.[264] Longer times (8–21 min) were found in a group of nine healthy dogs.[267]
4. When confounding variables are controlled, prolonged times indicate severe defects in the intrinsic and/or common pathways, including those caused by coagulation inhibitors (see PTT below).

B. Activated coagulation (clotting) time (ACT)

1. Point-of-care whole blood screening coagulation test of the *intrinsic* and *common* pathways
2. Principle and method (conventional visual method)
   a. Contact activation: Blood (2 mL) is drawn by atraumatic jugular venipuncture into a prewarmed (37°C) ACT vacuum tube containing silaceous (diatomaceous) earth, which activates the contact (intrinsic) pathway.
   b. The blood is mixed by five inversions and the tube is incubated (37°C) for 60 s.

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The tube is then checked (visually while tipping it) every 5 or 10 s for the first definite evidence of a clot.

c. The time from contact of blood with silaceous earth to the initial definite signs of a clot is the ACT.

d. Units: seconds (nearest 5 or 10 s, depending on frequency of inspection)

3. Interpretive considerations
   a. Interpretation is similar to PTT (see below), but the test is generally less sensitive.
   b. Severe thrombocytopenia (< 10,000 platelets/µL) is commonly stated to prolong ACT because of decreased phospholipid availability. However, there is very little documentation of this statement.[268,269]
   c. A standard protocol should be followed because modifications of the protocol may affect expected values. Variations have related to syringe versus vacuum collection, incubation temperature (various axillary temperatures versus a heating block), length of initial incubation period, and frequency of inspection for clots.
4. Prolonged ACT: indicates decreased *in vitro* function of the intrinsic or common pathways (see note to Table 5.7)
5. ACT may be used to monitor heparin therapy (target ACT ≈ 1.2 times baseline).
6. Automated point-of-care instruments are available and manufacturer recommendations should be followed.

IV. Activated partial thromboplastin time (PTT, APTT, aPTT)[270]

A. A screening coagulation test of the *intrinsic* and *common* pathways

B. Principle and method
   1. Contact activation: Citrated test plasma is incubated (37°C) with an excess of procoagulant phospholipids (partial thromboplastin) and a contact activator (e.g., kaolin, silicates, ellagic acid) to activate the intrinsic pathway via Factors XII and XI; the chelation of fCa^{2+} by citrate in the sample limits activation beyond Factor XIa.
      a. Partial thromboplastin consists of procoagulant phospholipids devoid of TF and therefore it is unable to activate the extrinsic pathway (Factor VII).
      b. In the original PTT assay, the glass tube (not added particulates) activated the intrinsic pathway.[271] In order to differentiate the two tests, the current test is often referred to as the activated partial thromboplastin time (APTT, aPTT). However, both tests require activation, and the original test is not routinely done.
   2. After a defined incubation time, a measured amount of prewarmed (37°C) $CaCl_2$ is added to counteract the effects of citrate and allow 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 $CaCl_2$ to clot detection is the PTT.*
   3. Point-of-care instruments are available and manufacturer recommendations should be followed.
   4. Units: seconds (usually reported to nearest 0.1 s, though reporting to the nearest half second or second may be more appropriate for some methods)

C. Interpretive considerations
   1. Laboratory-specific and species-specific reference intervals must be used because results vary substantially with variations in species, analyzers, reagents, and protocols. Also, values in healthy newborns (< 24 hr old) may be greater than reference intervals established for adults.[272,273]

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Table 5.7. Diseases or conditions that cause prolonged PTT

- Deficiencies of functional intrinsic or common pathway coagulation factors
  - Acquired (usually multiple defects)
    - Decreased production
      - [a]Hepatic disease (necrosis, cirrhosis, portosystemic shunt)
      - Vitamin K antagonism or deficiency
        - [a]Decreased vitamin K recycling in hepatocytes: anticoagulant rodenticide ingestion, coumadin overdose, moldy sweet clover ingestion (*Melilotus* spp.)
        - Decreased vitamin K absorption: biliary obstruction, infiltrative bowel disease, chronic oral antimicrobials, EPI
    - Increased coagulation factor inactivation/consumption
      - [a]DIC
      - Localized consumptive coagulopathy
    - Dilution of coagulation factors: acute, massive blood loss treated with crystalloid and colloid fluids, including Oxyglobin® solution
  - Hereditary (usually single defect)
    - Intrinsic pathway: PK, XII, XI, IX, VIII
    - Common pathway: X, V, II, I
- [a]Inhibition of intrinsic or common pathway coagulation factors: heparin, FDPs, antibodies to phospholipids, antibodies to coagulation factors

Note: ACT may also be prolonged in most conditions.

2. The test is relatively insensitive, but, for optimized assays, results should be prolonged when there is at least a 70% decrease in single factor activity. Milder reductions in individual factor activities may be detected when multiple factors are affected.
3. PTT assayed at 37°C may overestimate *in vivo* coagulation in markedly hypothermic patients because enzyme activities are temperature dependent.[274,275]
4. Lipemia, hemolysis, Oxyglobin® solution, and icterus interfere with PTT assays that detect clot formation optically.
5. Excessive citrate or improper handling (delayed processing, old sample, inappropriate temperature) may prolong PTT.

D. Prolonged PTT: Indicates decreased *in vitro* function of the intrinsic or common pathways (Table 5.7). Pure deficiencies of Factor XII or PK are typically not associated with clinical hemorrhage, but they are associated with prolonged PTT results in most assay systems.[250] Prolonged PTT resulting from PK deficiency may be corrected by increasing the incubation time of plasma with a particulate contact activator, or by using ellagic acid for contact activation.[276] Unexplained prolongations in PTT, without other evidence of impaired hemostasis, have been reported in cats.[277]

E. Shortened PTT is not reliable for detecting hypercoagulability, though increases in Factors V and VIII with inflammation may tend to shorten the PTT.

F. PTT is used to monitor heparin therapy (target PTT ≈ 1.5 times baseline).

V. Prothrombin time (PT), also called one-stage prothrombin time (OSPT) assay

A. A screening coagulation test of the *extrinsic* and *common* pathways

B. Principle and method

1. Test plasma and a Ca^{2+}-thromboplastin reagent (containing phospholipid and excess TF) are separately prewarmed (37°C).

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Table 5.8. Diseases or conditions that cause prolonged PT

Deficiencies of functional coagulation factors in the extrinsic or common pathway
[a]Acquired (usually multiple defects): see Table 5.7
Hereditary (usually single defect)
Extrinsic pathway: VII
Common pathway: X, V, II, I
[a]Inhibition of coagulation factors in the extrinsic or common pathway: heparin, FDPs, antibodies to phospholipids, antibodies to coagulation factors

2. A measured amount of prewarmed Ca^{2+}-thromboplastin reagent is forcibly added to a measured amount of prewarmed plasma to allow TF activation of Factor VII in the extrinsic pathway. Activation should proceed along the common pathway to result in formation of fibrin monomers, which polymerize to form an insoluble fibrin clot that can be detected optically or electromechanically, depending on the instrument. *The time from plasma-thromboplastin mixing to clot detection is the PT.*
3. Different thromboplastin reagents produce different results.[278] Some appear to be affected by PIVKA, which interfere with the generation of thrombin and inhibit coagulation *in vitro.*
4. Reagents and plasma may be diluted to lengthen PT and increase the sensitivity for detecting abnormalities.
5. Point-of-care instruments are available and manufacturer recommendations should be followed.
6. Units
   a. Seconds (usually reported to nearest 0.1 s, though reporting to the nearest half second may be more appropriate for some methods)
   b. May be reported as a unitless INR for monitoring warfarin therapy (see below)

C. Interpretive considerations: same as given above for PTT. Unless used to monitor warfarin therapy, PT values are best interpreted relative to a valid reference interval.

D. Prolonged PT: indicates decreased *in vitro* function of the extrinsic or common pathways (Table 5.8). PT is less sensitive to heparin than PTT, but it may be prolonged with certain thromboplastin reagents when blood contains therapeutic heparin concentrations.[279]

E. Shortened PT is not reliable for detecting hypercoagulability.

F. PT is used to monitor warfarin therapy (target PT $\approx$ 1.5–2.0 times baseline).

G. INR: unitless ratio (Eq. 5.1) used to help standardize the reporting of PT values to correct for differences in thromboplastin reagents among laboratories.[280] (In Eq. 5.1, reference PT is the mean of a valid reference interval for the species in question, not a random control sample value. The ISI [International Sensitivity Index] is a number determined and provided by the thromboplastin reagent manufacturer for each lot of reagent using a particular PT method. ISI, which reflects the relative sensitivity of the reagent to factor deficiencies, is determined by calibration against an international reference preparation. Thromboplastins with higher ISI values are less sensitive than thromboplastins with lower ISI values.)

$$\text{INR} = \left(\frac{\text{Patient PT}}{\text{Reference PT}}\right)^{\text{ISI}} \qquad \textbf{(5.1)}$$

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1. Developed and used for monitoring oral anticoagulant therapy (warfarin) in people
2. May allow more meaningful comparisons of PT results between laboratories but does not eliminate significant interlaboratory differences[281]
3. Examples using the INR
   a. Different patients and different thromboplastin reagents but the same PT value
      (1) Patient #1, ISI = 1.5: PT = 12.0 s; reference mean = 8.0 s (Eq. 5.2.a.)
      (2) Patient #2, ISI = 2.3: PT = 12.0 s; reference mean = 8.0 s (Eq. 5.2.b.)
      (3) Patients #1 and #2 had the same PT (12.0 s) when patient #2 was tested with a less sensitive thromboplastin reagent (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. 5.2.a.)
      (2) Patient #1, ISI = 2.3: PT = 10.3 s; reference mean = 8.0 s (Eq. 5.2.c.)
      (3) When Patient #1 was tested with the less sensitive reagent (greater ISI), the PT was only 10.3 s but the INR, and therefore degree of anticoagulation, was unchanged.

$$\text{Patient \#1 INR} = \left(\frac{12.0}{8.0}\right)^{1.5} = 1.8 \quad \textbf{(5.2.a.)}$$

$$\text{Patient \#2 INR} = \left(\frac{12.0}{8.0}\right)^{2.3} = 2.5 \quad \textbf{(5.2.b.)}$$

$$\text{Patient \#1 INR} = \left(\frac{10.3}{8.0}\right)^{2.3} = 1.8 \quad \textbf{(5.2.c.)}$$

4. A reliable reference mean for the species in question is required for valid use of the INR; this requirement mostly restricts its use to veterinary diagnostic laboratories. Substituting a control value for the reference mean is not acceptable.

VI. Thrombin time (TT), also called thrombin clotting time (TCT)
  A. Assesses the thrombin-induced conversion of fibrinogen to an insoluble fibrin clot
  B. Principle and method
    1. Thrombin is added to prewarmed test plasma (37°C); thrombin should cleave fibrinogen to form monomers, which should 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)
  C. Interpretive considerations: TT should be differentiated from TT_{Clauss}, which uses diluted plasma and high thrombin concentrations to give a better measure of functional fibrinogen concentration.[282] Lab-specific reference intervals should be used for interpretation because of method variations.[283]
  D. Prolonged TT[284]
    1. Hypofibrinogenemia or afibrinogenemia: increased consumption from localized coagulation or DIC, congenital deficiency (rare), possibly from decreased production resulting from hepatic failure; TT prolonged because of inadequate fibrinogen to form a fibrin clot[285-288]

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2. Dysfibrinogenemia: hereditary or acquired production of abnormal fibrinogen (rare); acquired form may occur with a variety of liver diseases; polymerization of fibrin impaired because of abnormal fibrinogen molecules[286-288]
3. Heparinized patient or sample: heparin interferes with thrombin activity via ATIII; can monitor heparinization with TT[283]
4. Increased [FDP]: interfere with fibrin polymerization and thrombin's action on fibrinogen;[289] can monitor fibrinolytic therapy with TT
5. Paraproteinemia: Some abnormal immunoglobulins from multiple myelomas may interfere with fibrin polymerization.[290]
6. Systemic amyloidosis (reported in people): Plasma from affected patients may contain an inhibitor of fibrin polymerization.[291]
7. Hyperfibrinogenemia: Rarely, in people, hyperfibrinogenemia has prolonged the TT, but the cause is not clear.[292,293]
8. TT is unaffected by decreased activity of any coagulation factor other than fibrinogen (including Factor XIII).

E. Shortened TT: It may occur with increased or activated clotting factors, or with soluble fibrin in the sample, but it is not a reliable measure of hypercoagulability.

VII. Fibrinogen

A. Fibrinogen activity (TT_{Clauss}):[282,284,294] a modification of the TT in which plasma is diluted so that fibrinogen concentration is rate-limiting for coagulation, and in which greater concentrations of thrombin are used to override much of the inhibition caused by heparin and FDP. TT_{Clauss} values of known fibrinogen concentrations are used to construct a reference (standard) curve from which test plasma [fibrinogen] can be estimated.
1. TT_{Clauss} is inversely proportional to [fibrinogen] when high concentrations of inhibitors and dysfibrinogenemia are absent.
2. TT_{Clauss} is measured in seconds, but values for [fibrinogen] are read from the reference (standard) curve and reported in units of mg/dL.
3. Heparin will not interfere at clinically therapeutic concentrations in human samples, but it will interfere at high concentrations occurring after bolus injection, with inappropriate blood collection from a heparinized catheter, or with blood collected into a heparinized tube.
4. FDP have a minimal effect on TT_{Clauss} in human samples unless present at high concentrations concurrently with hypofibrinogenemia.

B. Fibrinogen antigen: Antifibrinogen antibodies are used to detect fibrinogen antigen by immunoassay. Prolonged TT and TT_{Clauss} with decreased fibrinogen antigen supports hypofibrinogenemia. Prolonged TT and TT_{Clauss} without decreased fibrinogen antigen supports dysfibrinogenemia (rare).

C. Fibrinogen concentration by heat precipitation: This technique is not precise or accurate enough for use in hemostasis testing.

D. Interpretive considerations: Plasma [fibrinogen] reflects the balance between production and consumption, not production or consumption alone. The accelerated consumption of fibrinogen during hypercoagulable states may be masked by increased production associated with inflammation or pregnancy.[116,295] Values in healthy newborns (< 24 hr old) may be lower than reference intervals established for adults.[273,296]

VIII. Other specific coagulation factors

A. Other specific coagulation factor assays may be used in specialized veterinary laboratories

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Table 5.9. Hereditary coagulation factor deficiencies reported in animals

Pathway Factor	Condition	Expected coagulation test results PTT	PT	TT
Extrinsic				
VII	Factor VII deficiency	WRI	↑	WRI
Intrinsic				
PK	Prekallikrein deficiency	↑	WRI	WRI
XII	Hageman trait	↑	WRI	WRI
XI	Factor XI deficiency	↑	WRI	WRI
IX[a]	Hemophilia B	↑	WRI	WRI
VIII[a]	Hemophilia A	↑	WRI	WRI
Common				
X	Factor X deficiency	↑	↑	WRI
V[b]	Factor V deficiency	↑	↑	WRI
II	Hypoprothrombinemia	↑	↑	WRI
I	Hypofibrinogenemia or afibrinogenemia	↑	↑	↑
Combined				
II,VII,IX,X	Vitamin K-dependent coagulation factor deficiency	↑	↑	WRI

[a] X-linked recessive transmission; rarely occurs in females.
[b] May be thrombocytopenic or have prolonged BMBT.

if screening tests and clinical findings suggest factor deficiencies; they may be used to characterize acquired deficiencies but usually are done to identify hereditary deficiencies[286,297,298] (Table 5.9).

B. Factor deficiencies are indicated if dilution of test plasma with normal plasma corrects prolonged PT or PTT (provides missing factor), whereas failure to correct coagulation times supports the presence of an inhibitor.

C. Clotting assays for specific factors: assess the ability of the patient plasma to correct the PT or PTT of specific factor-deficient plasmas[299]
   1. The PTT (used for evaluating intrinsic pathway factor deficiencies) or PT (used for evaluating extrinsic or common pathway factor deficiencies) of the known factor-deficient plasma should correct with addition of diluted patient plasma if the patient plasma contains the missing factor. However, the PTT or PT will not correct if the patient plasma has the same factor deficiency.
   2. To determine the amount of factor activity in the test plasma, a reference curve (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. Using the reference curve, observed clotting times for dilutions of the test plasma are converted to units of percent activity relative to the reference plasma pool. The reference plasma pool is considered to have 100% activity (units of U/dL or U/mL relative to the plasma pool with activity of 100 U/dL or 100 U/mL, respectively, are also used).

D. Chromogenic assays: assess the ability of a factor or cofactor to lead to enzymatic cleavage of a substrate, thus producing a detectable color change that is proportional to the amount of factor or cofactor present[300]

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IX. Proteins induced by vitamin K antagonism or absence (PIVKA)

A. PIVKA are the incompletely gamma-carboxylated vitamin K-dependent coagulation factors (e.g., des-γ-carboxy prothrombin, often referred to as PIVKA-II because prothrombin is coagulation Factor II) that are produced by hepatocytes during periods of vitamin K antagonism, absence, or deficiency (see Major Bleeding Disorders, II.B below).[237] 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.

B. 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.[301] Charge differences between PIVKA and their carboxylated forms also allows for detection by high-performance liquid chromatography.[302]

C. Increased plasma [PIVKA] may occur with vitamin K deficiency, vitamin K antagonism, or as a result of production by malignant hepatocytes in human patients with hepatocellular carcinomas.[303]

D. 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].[304,305] It is a PT test that detects decreased activities of coagulation Factors II, VII, and X from any cause. Factor V and fibrinogen are not assessed because they are provided in the assay. In addition, 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. PIVKA do not appear to interfere with functional factors *in vivo*.[306]

1. The Thrombotest PT assay is sensitive to abnormalities induced by anticoagulant rodenticides in dogs, but it also detects other acquired coagulopathies and hereditary Factor VII deficiency of beagles.[278,307,308]
2. Prolonged Thrombotest PT values were present in cats with a variety of hepatic or inflammatory bowel diseases, most without clinical evidence of a bleeding tendency.[304]
    a. In a subset of these cats treated with vitamin K_1, Thrombotest PT values were WRI 3–5 days after treatment was instituted, indicating that prolonged Thrombotest PT values were at least transiently vitamin K responsive.
    b. While the assay appears sensitive in cats, its specificity for clinically significant bleeding tendencies is unknown.

X. Russell viper venom time (RVVT): The test is not widely available. Direct activation of Factor X by venom of the Russell's viper leads to a clot; prolonged clotting times indicate an abnormality of the common pathway (Factors X, V, II, or I).

XI. Endogenous anticoagulants

A. Antithrombin III (ATIII): assayed to provide information about a patient's anticoagulant status

1. Plasma ATIII is usually measured by chromogenic (functional) assays rather than immunoassays that detect ATIII antigen but not function.[282]
2. Principle and method: Test plasma is added to a reagent containing heparin and excess thrombin or excess Factor Xa and also containing the corresponding chromogen-labeled substrate for thrombin or Factor Xa. The more ATIII present in

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the test plasma, the less activity of thrombin or Factor Xa, and therefore the less color change, which is measured spectrophotometrically.

3. Units: percent activity compared to a species-specific plasma pool considered to have 100% activity (has been similarly expressed as percent activity compared to human plasma, in which case the results may vary considerably because of species differences in ATIII activity).[309-311] ATIII also has been reported in U/mL or U/dL, where 1 U/mL or 100 U/dL, respectively, are arbitrarily assigned as the mean values of the reference sample pool.
4. Stability: ATIII appears to be stable in citrated plasma for 6 months at -70°C and for 6 weeks in whole blood stored at 4°C.[311]
5. Interpretive considerations
    a. Foals and human neonates have been shown to have plasma ATIII activities that are substantially lower than adult values.[296,312] This must be considered when interpreting ATIII values in young patients, especially in the absence of age-matched reference intervals.
    b. ATIII activity may be overestimated when measured by some thrombin chromogenic assays because heparin cofactor II activity may be detected in addition to ATIII activity.[311,313]
6. Decreased ATIII activity[309,311,314] (Fig. 5.5)
    a. Decreased production
        (1) Inherited deficiencies: occur in people as type I (decreased antigen and activity, a quantitative disorder) and type II (decreased activity but normal amounts antigenically, a qualitative disorder);[315] not reported in animals
        (2) Liver disease (including portosystemic shunts):[316] may cause or contribute to ATIII deficiency in several ways, including decreased production related to decreased hepatocellular mass
        (3) Inflammation: ATIII was shown to be a negative acute phase protein in human liver cells and in baboons,[317] although increased ATIII activity has been suggested to reflect a positive acute phase response in rabbits[318] and cats.[179,316,319]
        (4) Estrogens: may contribute to ATIII deficiency by causing a mild to moderate decrease in ATIII synthesis[312,320]
    b. ATIII loss: Like albumin, ATIII may be lost from the body in animals with protein-losing nephropathy and, possibly, with protein-losing enteropathy;[321] urinary loss of ATIII without a concurrent impairment of coagulation contributes to severe thrombotic disease in nephrotic syndrome patients, though other factors are involved.[322]
    c. Increased hepatic clearance of ATIII-enzyme complexes
        (1) Localized or disseminated consumptive coagulation states cause decreased plasma ATIII activities when hepatic clearance of ATIII-enzyme complexes exceeds ATIII production;[282,311,316,323] decreased ATIII activity may be either a cause or an effect of hypercoagulable states.
        (2) Heparin therapy: accelerates the use and, therefore, hepatic clearance of ATIII, leading to decreased plasma activity;[324,325] because ATIII is required for heparin's full anticoagulant effects, patients with subnormal ATIII activities are expected to have diminished responses to heparin and may be heparin resistant.[326]
7. Increased ATIII activity

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a. Of unknown diagnostic utility, but not considered a problem
b. Cortisol: Mildly to moderately increased ATIII synthesis has been associated with exogenous cortisol administration in dogs,[320] but dogs with hyperadrenocorticism had decreased ATIII activity (evidence for a hypercoagulable state).[327]
c. Inflammation: may increase ATIII synthesis as part of the positive acute phase response in some species, including cats.[179,316,318,319]
d. Cats made taurine deficient had greater plasma ATIII activities than they had prior to being fed the taurine-deficient diet,[179] and cats with cardiac disease and hyperthyroidism had increased ATIII activities.[319] A role for thyroid hormones in increasing plasma ATIII activities in cats has not been reported but could be considered.[328-330]

8. Thrombin-antithrombin III (TAT) complexes: [TAT] is measured to assess for activation of coagulation, especially latent coagulation that is not otherwise apparent. Increased plasma [TAT] indicates an increase in local or systemic thrombin generation, which is associated with thromboembolic disease or hypercoagulable states.[331-333] Decreased hepatic clearance of TAT complexes could theoretically contribute to increased plasma [TAT]. Increased [TAT] may occur without appreciable decreases in ATIII activity.
   a. Equine and canine plasma TAT complexes have been measured with a human ELISA, primarily for research purposes.[327,334,335] [TAT] is reported in units of μg/L.
   b. TAT complexes may readily form *in vitro* if collection techniques are poor and coagulation occurs.[336]

B. Protein C: assayed to provide information about a patient's anticoagulant status; low plasma concentrations or activities predispose to thrombosis
   1. Protein C is assessed infrequently in veterinary medicine, mostly for horses. Antigen concentration can be measured immunologically,[337] or activity can be measured by clot-based or amidolytic-based functional assays.[338] Antigenic assessment does not detect functional deficiencies, including those induced by vitamin K antagonism or deficiency. Results of functional assays vary with the method, and species-specific modifications may be required.[339,340] Results are reported as percent activity or antigen relative to a reference plasma pool considered to have 100% activity or antigen. Protein C antigen (not activity) values in healthy neonates (< 24 hr old) may be lower than reference intervals established for adults.[272,273]
   2. Decreased protein C activity may occur with hereditary deficiencies (type I, antigenic; or type II, functional[341]), consumptive coagulation,[342] vitamin K antagonism or deficiency (protein C is vitamin K-dependent), decreased protein S (cofactor of protein C, which also is vitamin K-dependent), liver disease, antiphospholipid-protein antibodies that inhibit the function of protein S or protein C directly, or with abnormalities of Factor V.[315,340,343] Protein C activity (not antigen) values in healthy neonates (< 24 hr old) may be greater than reference intervals established for adults.[272,273]

FIBRINOLYSIS

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

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a secondary hemostatic plug. Fibrinolysis is normally localized to the hemostatic plug. When coagulation occurs, plasminogen (inactive zymogen) binds to the forming fibrin. t-PA released from stimulated endothelial cells binds to the fibrin-plasminogen complex and proteolytically cleaves plasminogen to form plasmin. Plasmin enzymatically degrades fibrin, fibrinogen, Factors Va and VIIIa, vWf, and other prothrombotic factors. Plasmin released into circulation is rapidly inhibited, primarily by α_2-antiplasmin. The activation of plasmin is promoted by protein Ca and inhibited by PAI, which protein Ca inactivates.

B. Plasmin degradation of fibrin and fibrinogen produce FDP (Fig. 5.7), which can be measured to assess fibrinolysis.

C. FDP appear to be cleared from circulation by the liver (hepatocytes and macrophages) and kidneys (via catabolism or excretion).[344-347] Nonhepatic parts of the MPS may also be involved.[348,349]

II. Fibrin or fibrinogen degradation products (FDP)

A. Purpose: to detect increased fibrinolysis associated with excessive coagulation; also detects increased fibrinogenolysis

B. Sample: serum or plasma

1. Serum assays: Blood is collected into special tubes containing thrombin or *Botrox atrox* venom (reptilase), either of which can be used to consume fibrinogen via coagulation. Soybean trypsin inhibitor or aprotinin are present to inhibit plasmin and therefore *in vitro* FDP formation.

Fig. 5.7. Schematic representation of the formation of cross-linked fibrin from fibrinogen (*thin arrows*) and of the plasmin-mediated degradation of fibrinogen and fibrin to form FDP (*thick arrows*).

- *Fibrin formation:* Fibrin is formed from fibrinogen, an elongated molecule that has a central E region and peripheral D regions. When thrombin is generated, it cleaves fibrinopeptides A and B from the E region of fibrinogen to form fibrin monomers. Unlike fibrinogen, fibrin monomers can polymerize (noncovalently) to form protofibrils of two or more strands (two shown here). Thrombin also activates Factor XIII to Factor XIIIa, which cross-links adjacent D regions of different fibrin monomers to form stable cross-linked fibrin protofibrils. These can associate to form larger fibrin fibers (not shown) and a stable clot or thrombus.
- *FDP formation:* Plasmin cleaves fibrin and fibrinogen at specific sites to form several fibrin and fibrinogen fragments called fibrin and fibrinogen degradation products, collectively referred to as FDP.
  - o Fibrinogen: Fibrinogen is cleaved to form Fragment X and smaller fragments referred to as $B\beta_{1\text{-}42}$ and $A\alpha$ polar appendages. Fragment X is further degraded to fragments D and Y, and fragment Y is degraded to fragments D and E.
  - o 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 fibrinogen during the formation of fibrin. Fibrin monomers and non-cross-linked protofibrils are degraded to form fragment X′ and smaller fragments $B\beta_{15\text{-}42}$ and $A\alpha$ polar appendages. Fragment X′ is further degraded to fragments D and Y′, and Fragment Y′ is degraded to fragments D and E′.
  - o Cross-linked fibrin: Plasmin-mediated degradation of cross-linked fibrin produces a different set of FDP because of the covalent bonds formed by Factor XIIIa between adjacent D regions. Initial degradation of cross-linked fibrin yields the large fragments DD/E′, Y′D/DY′, and Y′Y′/DX′D. These are further degraded to give a variety of smaller FDP including D-dimers, which can be measured.
  - o Antigenic similarities of FDP produced from fibrinogen and fibrin make the current FDP assay nonspecific for fibrinogenolysis or fibrinolysis (with or without cross-linking). However, D-dimer assays allow specific detection of fibrinolysis after fibrin cross-linking, and increased values therefore indicate that there has been both coagulation and fibrinolysis.

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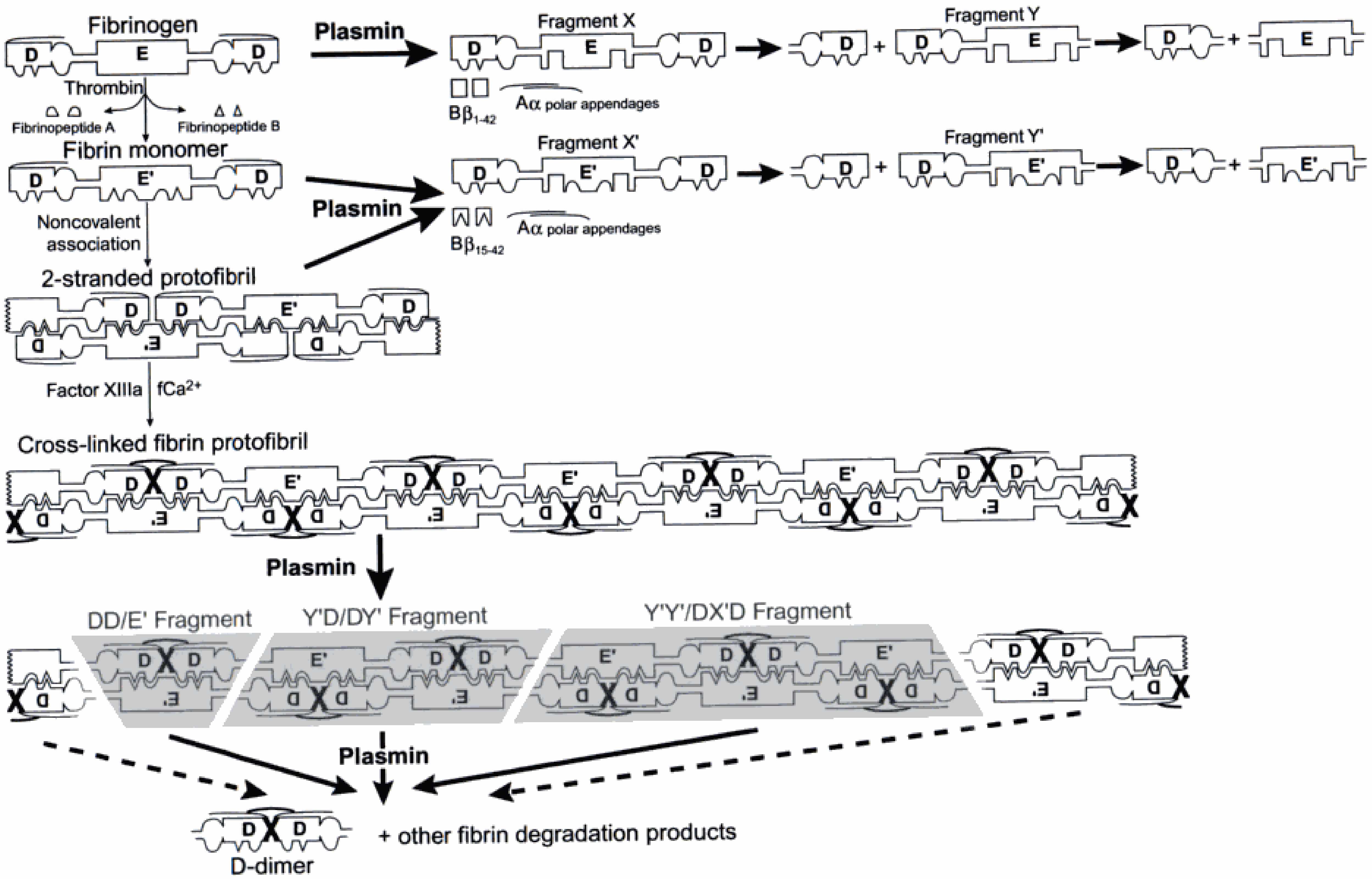

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2. Plasma assays: Blood is collected as for screening coagulation tests (see Coagulation, II above); citrated plasma is used.
3. As for other tests of hemostasis, care must be taken to prevent or minimize coagulation during collection and processing. When plasma is the test sample, clotted samples should be discarded.
4. Stability: often recommended to test plasma within 24 hr when stored at 4°C and within 20 days when frozen at -20°C

C. Analytical concepts
1. Assays are latex agglutination immunoassays.[350] Dilutions of test serum or plasma are incubated with latex beads coated with antibodies to human FDP; agglutination of the beads indicates increased FDP.
2. Antibody specificity
   a. The specific reactivity of assay antibodies with different FDP fragments from veterinary species is not known, but cross-reactivity of some polyclonal reagents with canine FDP has been shown.[351] The sensitivity and specificity of various assays in the detection of FDP in various species is also not known, but clinical correlations with disease support that there is at least partial cross-reactivity.
   b. Plasma assay: uses a monoclonal antibody to FDP that does not react with fibrinogen, thus allowing use of plasma (which contains fibrinogen) as the test sample
   c. Serum assay: uses a polyclonal antibody to FDP that also binds fibrinogen, thus necessitating the *in vitro* removal of fibrinogen with reptilase or thrombin
3. Unit: usually μg/mL (may also be reported in ng/mL or μg/L); reported semiquantitatively as (1) less than a low cutoff (e.g., < 5 μg/mL with plasma tests, < 10 μg/mL with serum tests; considered normal), (2) increased (5–20 μg/mL with plasma tests, 10–40 μg/mL with serum tests), or (3) increased more (> 20 μg/mL with plasma test, > 40 μg/mL with serum test)
4. Interpretive considerations
   a. Prozone: With high [FDP], agglutination may occur at the greater dilution but not at the lesser dilution.
   b. False results: Serum assay results may be falsely decreased by FDP incorporation into the clot as it forms.[352] They may be falsely increased by incomplete removal of fibrinogen from the sample due to the presence of dysfibrinogenemia (rare) or heparin (if thrombin rather than reptilase is used to activate clotting).[353] Generation of FDP during blood collection can cause false increases with plasma or serum assays.
   c. Values in apparently healthy neonates may be greater than reference intervals established for adults.[272,273]
   d. Values in apparently healthy elderly people are often increased, probably related to changes in clearance rates or to an increased incidence of occult disease.[354]

D. Increased [FDP]
1. Occurs in numerous different diseases and conditions in which at least one of the following is present:[355]
   a. Increased fibrinolysis
      (1) Localized intravascular coagulation (e.g., isolated thrombosis or thromboembolism)
      (2) Disseminated intravascular coagulation
      (3) Internal hemorrhage in body cavities or tissues: Bloody fluid collected from body cavities after hemorrhage contains high concentrations of FDP, and

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increased blood [FDP] have been measured in such affected patients.[356] Cause and effect can be difficult to determine, but hemorrhage into body cavities or tissues may cause increased blood [FDP]. However, 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.[357]

b. Increased fibrinogenolysis: requires hyperplasminemia or high local concentrations of plasmin; not specifically assessed in veterinary medicine; may arise with certain envenomations;[106] occurs from administration of plasminogen activators (e.g., t-PA);[358] may accompany fibrinolysis associated with thrombotic disease;[359] may arise from excessive endothelial release of t-PA, for which causes are not clearly defined but may include hypotensive shock, surgical trauma, and heatstroke[360]

c. Decreased FDP clearance: Decreased hepatic clearance may contribute to the increased [FDP] in some patients with liver disease.[347] Decreased renal function may contribute to increased [FDP] in some patients with renal failure.[344] Theoretically, decreased MPS activity could contribute to increased [FDP].

2. Effects of increased [FDP] on other hemostatic functions and tests: PT, PTT, TT, ACT, and WBCT may be prolonged and platelet function may be impaired because FDP have anticoagulation and antiplatelet effects. They compete with fibrinogen for the active site of thrombin, and thus may inhibit the conversion of fibrinogen to fibrin.[361,362] They compete with fibrinogen for platelet binding sites, and thus may inhibit platelet aggregation.[363] They associate with fibrin monomers and may disrupt normal polymerization.[289,364]

III. D-dimer

A. Purpose: to detect increased fibrinolysis secondary to excessive coagulation; does not detect fibrinogenolysis because antibodies react only with FDP formed from cross-linked fibrin (Fig. 5.7)

B. Sample

1. Usually citrated plasma collected and handled as described above 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 loss of D-dimers into the clot may occur.

2. Stability: It is recommended to test plasma within 24 hr when stored at 4°C and within 20 days when frozen at -20°C.[365]

C. Analytical concepts

1. Assays detect D-dimers or x-oligomers, which are larger fragments of plasmin-digested fibrin containing the D-dimer antigen.[366]

a. Assays developed for human D-dimer measurement are immunoassays. They vary in method, sample, and antibody used. Their utility in animals, which is being evaluated, varies with assay and species.[365,367]

b. Immunoturbidimetric (quantitative) and latex agglutination tests (semiquantitative) have been evaluated or used in animals.[365,366]

2. Antibody specificity: specificity of the test antibodies for D-dimers of veterinary species has not been proven

3. Unit: µg/mL, ng/mL, or µg/L

D. Increased plasma [D-dimer]: Interpretation is similar to that for increased [FDP], except that values should reflect only increased fibrinolysis (not fibrinogenolysis) or decreased

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clearance of fibrin degradation products (not fibrinogen degradation products) by the liver or MPS.[368,369]

IV. Other assays may be used to measure fibrinolytic components (e.g., plasminogen, α_2-antiplasmin, t-PA, plasminogen activator inhibitor-1, fibrinopeptide A) or fibrinolytic activity (euglobulin lysis time test, thromboelastography), but they are not widely used in clinical settings and will not be discussed here.

BLOOD VESSELS (ENDOTHELIAL CELLS)

Although blood vessels (especially the endothelial cells) are very important in maintaining normal hemostasis, their assessment in the clinical pathology laboratory is limited primarily to tissue biopsy and histologic evaluation (e.g., for vasculitis or thrombosis). Vascular lesions are usually obvious when hemorrhage is the result of surgical or accidental vascular trauma. Diseases involving small vessels, such as RMSF and equine purpura hemorrhagica, may be associated with petechiae and ecchymoses caused by combinations of direct vascular damage, thrombocytopenia, and thrombopathia. Hemostasis testing may help exclude other disorders, but it will not provide a specific diagnosis.

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 (e.g., gross examination, imaging, serology, biopsy), but hemostatic tests are useful to exclude primary blood disorders and may provide diagnostic clues because of secondary changes in blood constituents (e.g., thrombocytopenia with vasculitis)
         a. Large vessels: surgical or nonsurgical trauma, invasion, aneurysms, anomalies
         b. Small vessels: vasculitides (infectious, immune-mediated, chemical), vasculopathies (rare)
      2. Blood disorders: typically identified by hemostatic tests
         a. Impaired primary hemostasis: thrombocytopenia, thrombopathia, vWD
         b. Impaired secondary hemostasis: hereditary or acquired defects in coagulation
         c. Excessive fibrinolysis: DIC, some envenomations
   B. Clinical findings: essential for establishing a final diagnosis, but the bleeding pattern, breed, and age may be of particular value in directing diagnostic plans
      1. Bleeding pattern
         a. Petechiae and ecchymoses should prompt consideration of severe thrombocytopenia or thrombopathia, though 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 and ecchymoses are absent. However, hematomas may form with platelet defects.
         c. Hemorrhage through mucosal surfaces (e.g., epistaxis, gastrointestinal hemorrhage, prolonged estral bleeding) may occur with thrombocytopenia, thrombopathia, vWD, or defects in coagulation. Prolonged hemorrhage secondary to venipuncture or surgical or nonsurgical trauma is also not specific.

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Table 5.10. Possible causes of abnormal results for the major tests of hemostasis

Test or analyte	Result	Possible causes
ACT	↑ time	Defect in intrinsic and/or common pathways
ATIII	↓ activity[a]	Renal loss, consumptive coagulation, or decreased production (become hypercoagulable)
BMBT	↑ time	Thrombocytopenia, thrombopathia, vWf deficiency, anemia
Clot retraction	↑ time	Thrombocytopenia, thrombopathia
Coagulation factors	↓ activity	Hereditary or acquired factor deficiencies (hypocoagulable state)
D-dimers	↑ concentration	Fibrinolysis (secondary to coagulation), decreased clearance
FDP	↑ concentration	Increased fibrin(ogen)olysis, decreased clearance
Fibrinogen[b]	↓ concentration	Decreased hepatic production, increased consumption (may be hypocoagulable)
	↑ concentration	Inflammation, dehydration
Protein C	↓ activity[c]	Decreased hepatic production, abnormal production (vitamin K antagonism or absence), loss, consumption (may be hypercoagulable)
PT	↑ time	Defect in extrinsic and/or common pathways
PTT	↑ time	Defect in intrinsic and/or common pathways
RVVT	↑ time	Defect in common pathway
TT	↑ time	Hypofibrinogenemia, dysfibrinogenemia, heparinized patient or sample, increased FDPs
vWf:Ag	↓ concentration	vWD or vWD carrier
	↑ concentration	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, fibrinogen concentrations are usually determined from TT_{Clauss} values; TT_{Clauss} values are inversely correlated with fibrinogen concentrations unless prolonged by high concentrations of heparin or high concentrations of FDPs when fibrinogen concentrations are low.

[c] Chromogenic assays assess activity, but immunologic assays assess concentration; concentration may be WRI while activity is decreased if there is abnormal production of protein C (e.g., vitamin K antagonism or deficiency).

2. Breed and age
   a. Breed predispositions for inherited diseases may help in selection of appropriate diagnostic tests.[370]
   b. Hemorrhage in a young animal should prompt consideration of an inherited defect of platelets (Table 5.4), vWf, or coagulation factor deficiency (Table 5.9) if another cause is not apparent. Molecular genetic testing may be available for some characterized mutations in some affected and carrier animals (e.g., vWD).[371]

C. Hemostasis tests: A summary of available screening and specialized hemostasis tests is shown in Table 5.10 to aide in selection and interpretation of specific tests.

D. Major patterns of hemostasis test results: Hemostasis in the bleeding patient is best evaluated by multiple tests (hemostasis profiles). Examples of the major patterns of common hemostatic test results in bleeding patients are shown in Table 5.11.
   1. Pattern 1: Prolonged BMBT without thrombocytopenia or severe anemia suggests vWD or a thrombopathia. vWf analysis or platelet function studies may be indicated.

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Table 5.11. Interpretation of the major patterns of common hemostasis test results

Pattern	PT	PTT[a]	FDP[b]	Fibrinogen[c]	Platelets	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[f]
4	↑	WRI	WRI	WRI	WRI	WRI	Factor VII deficiency[f]
5	↑	↑	WRI	WRI	WRI	WRI	Common pathway defect or multiple defects
6	↑	↑	WRI	↓	WRI	WRI-↑	Dysfibrinogenemia or afibrinogenemia
7	↑	↑	↑	↓	↓	↑	Fulminant DIC[g]

[a] ACT would mirror PTT results in most diseases but may be less sensitive and therefore WRI.
[b] Plasma [FDP] may increase with hemorrhage into body cavities and tissues (see text).
[c] For assessment of hemostasis, fibrinogen concentrations are usually determined from TT_{Clauss} values; TT_{Clauss} values are inversely correlated with fibrinogen concentrations unless prolonged by high concentrations of heparin or high concentrations of FDPs.
[d] See text for expanded interpretive comments.
[e] PTT may occasionally be increased with canine vWD if Factor VIII is concurrently deficient.
[f] Pattern may also occur with early stages of hepatic disease or vitamin K antagonism or deficiency.
[g] Pattern only applies to severe, fulminant form of DIC.

Specifically defining thrombopathias may be difficult. PTT may occasionally be normal or prolonged with canine vWD if Factor VIII is concurrently deficient enough.

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. 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 PTT is indicative of an intrinsic pathway defect, though insensitivity of the PT assay may mask other abnormalities.
   a. Acquired: Hepatic disease; heparin contamination of the sample (e.g., inappropriate collection from a heparinized catheter) or heparin therapy should be considered.
   b. Inherited: Hemophilia A and B caused by deficiencies of Factor VIII and Factor IX, respectively, are most common (extremely rare in females because they are X-linked recessive traits) and may be associated with severe hemorrhage. Deficiencies in Factor XII are not associated with hemorrhage. PK deficiencies are associated with little or no hemorrhage. Deficiency of Factor XI is associated with mild hemorrhage, usually in response to trauma.
4. Pattern 4: Isolated prolongation of PT is indicative of an extrinsic pathway (Factor VII) defect.
   a. Acquired: Vitamin K antagonism or deficiency should be considered. Factor VII has the shortest half-life of the vitamin K-dependent factors, and three of the five factors in the extrinsic/common pathway are vitamin K-dependent. Therefore, the PT may be affected by vitamin K antagonism or absence before the PTT, especially when using a PIVKA-sensitive PT method.

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b. Inherited: Factor VII deficiencies are rare and associated with mild hemorrhage, usually in response to 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, which would be associated with hypofibrinogenemia).
   a. Acquired: Combined deficiencies of vitamin K-dependent factors (II, VII, IX, X) caused by vitamin K antagonism or deficiency, in which case hemorrhage may be severe and thrombocytopenia may be present. Increases in [fibrinogen] and decreases in [FDP] have been reported in people receiving warfarin, and may occur in other species. Hepatic disease should also be considered, especially if fibrinogen concentration or activity is low-normal. Variations of this pattern seen with hepatic disease include combinations of increased [FDP], decreased [fibrinogen], thrombocytopenia, and prolonged BMBT.
   b. Inherited: Rare inherited Factor X or Factor II 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 (Devon rex cats).
6. Pattern 6
   a. Acquired: Hepatic disease is a consideration for this pattern (see discussion of pattern 5). Consumptive coagulation (e.g., DIC) could also be considered, but thrombocytopenia and increased [FDP] are usually expected with DIC.
   b. Inherited: A marked decrease in fibrinogen concentration or activity along with prolonged PT and PTT should prompt consideration of rare inherited dysfibrinogenemia or afibrinogenemia. 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. Findings with hepatic failure could be similar.

II. Pathogenesis: The pathogeneses of hemorrhagic defects in primary hemostasis have been discussed. Inherited disorders of secondary hemostasis are the result of various genetic alterations that result in decreased, absent, or abnormal production of hemostatic factors. Acquired bleeding disorders often have a more complex pathogenesis. The pathogeneses of hemostatic abnormalities in selected acquired bleeding disorders are discussed below.
   A. Hepatic disease[277]
      1. Bleeding is uncommon but may occur if hepatic failure is severe or if it is associated with DIC. Other laboratory evidence of hepatic failure would be expected in a patient bleeding because of the hepatic failure.
      2. Abnormal hemostatic test results and their potential causes
         a. Prolonged PT, PTT, ACT, TT: decreased hepatic production of coagulation factors because of decreased functional hepatic mass (including portosystemic shunts[372]); abnormal production of vitamin K-dependent coagulation factors because of decreased vitamin K absorption secondary to cholestasis or, possibly, decreased food intake
         b. Increased [FDP]: decreased hepatic clearance of FDP and plasminogen activators; increased FDP production (fibrinolysis) because of accompanying intravascular coagulation, which is sometimes disseminated

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c. Thrombocytopenia: increased splenic sequestration resulting from portal hypertension and splenomegaly; consumption secondary to accompanying intravascular coagulation
d. Prolonged BMBT: thrombocytopenia; acquired thrombopathia
e. Decreased [fibrinogen]: decreased hepatic production; consumption secondary to accompanying intravascular coagulation
f. Decreased ATIII: decreased hepatic production; consumption secondary to accompanying intravascular coagulation
g. Thrombopathia: increased [FDP]; unknown causes

3. Results of routine hemostatic tests are not good predictors of surgical hemorrhage in human patients with hepatic disease[373] or of hemorrhage induced by collecting liver biopsies.[287] This is likely because the net balance of all antithrombotic and prothrombotic processes is not altered as much as the function of the isolated processes that are tested.

B. Vitamin K antagonism or deficiency
1. Vitamin K antagonism: Bleeding is common.
a. Vitamin K antagonists may be ingested in several forms.
(1) Anticoagulant rodenticides, e.g., coumarin, warfarin, pindone, indandiones, bromadiolone, brodifacoum, and diphacinone; specific rodenticides may be detected by high performance liquid chromatography.
(2) Sweet clover or sweet vernal grass containing bishydroxycoumarin, a metabolite produced from the actions of certain molds (cattle)[374,375]
(3) Warfarin therapy: overdose, or displacement from plasma proteins by additional protein-binding drugs (e.g., phenylbutazone)
(4) Excess sulfaquinoxaline, a coccidiostat with vitamin K antagonistic effects[376-378]
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 (Fig. 5.8).[237] When normal coagulation factors in the circulation ($t^1/_2$ = 6–40 hr) decrease enough, coagulation times become prolonged (within 24 hr)[379] and hemorrhage may occur.

2. Vitamin K deficiency: rarely deficient enough to cause hemorrhage
a. Vitamin K is absorbed in the intestine after ingestion or production by intestinal bacteria.
b. 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 (normal diets contain excess vitamin K) may cause or contribute to vitamin K deficiency.[380,381]
(2) Gut sterilization by antimicrobials may cause or contribute to vitamin K deficiency.[380,382]
(3) Malabsorption of vitamin K (a fat-soluble vitamin)[304]
(a) Intrahepatic or extrahepatic cholestasis (decreased fat digestion and absorption, therefore decreased vitamin K absorption)
(b) Intestinal malabsorption diseases (e.g., infiltrative bowel disease)
(c) EPI (decreased fat digestion and absorption, therefore decreased vitamin K absorption)
c. Diminished but not absent vitamin K may lead to a subclinical mixture of normal coagulation factors and PIVKA. Vitamin K supplementation was associated

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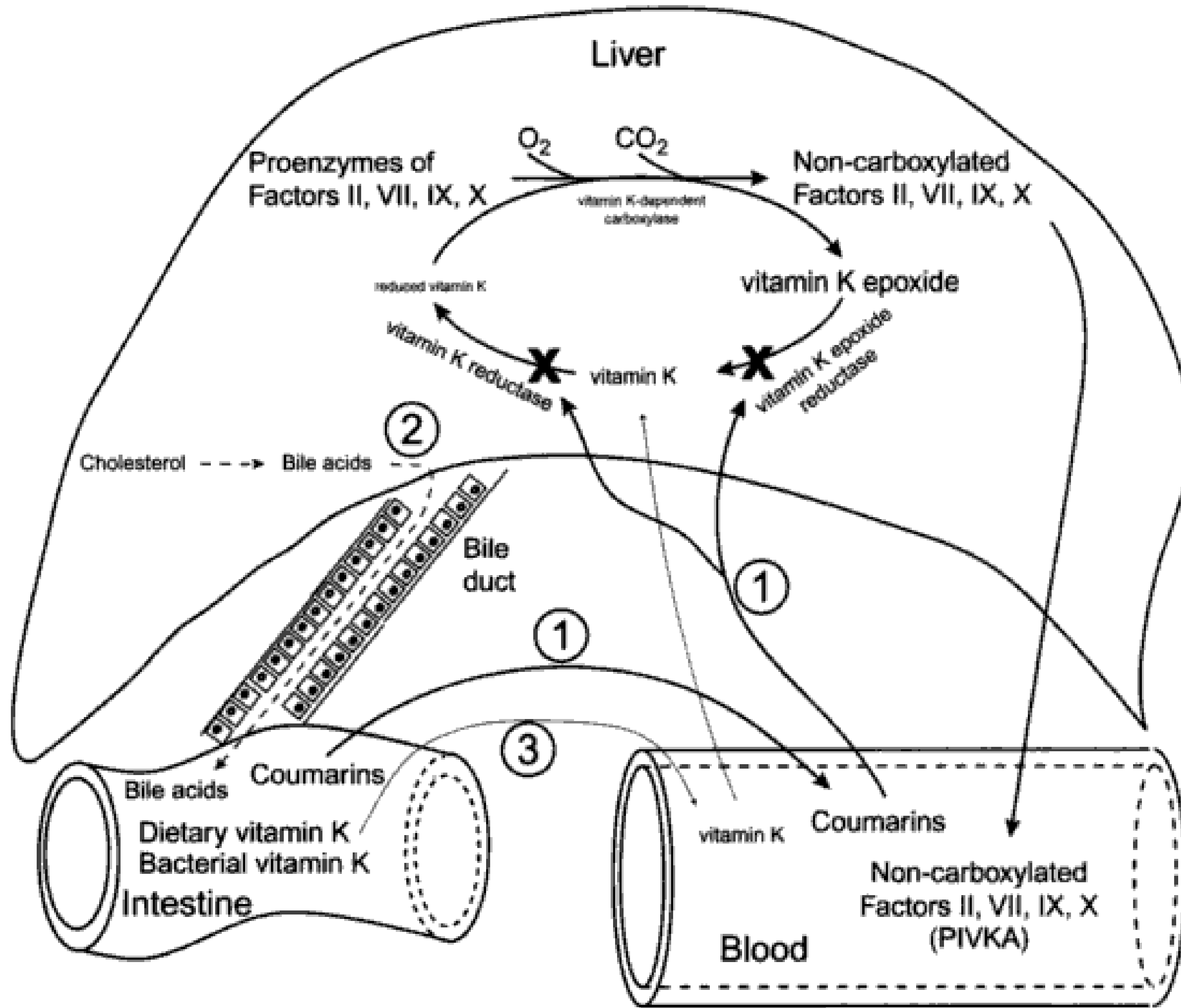

Fig. 5.8. Effects of vitamin K antagonism or deficiency on hepatocyte production of vitamin K-dependent coagulation factors (Factors II, VII, IX, and X). Circled numbers in the figure denote the three following processes that result in production of defective Factors II, VII, IX, and X.

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, the form of vitamin K required for the vitamin K-dependent carboxylase enzyme to carboxylate the coagulation proteins. 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, however, and they enter the blood and circulate in a permanently nonfunctional state. These non-carboxylated 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).
2. *Deficiency due to cholestasis (intrahepatic or posthepatic):* Impaired bile flow results in impaired fat absorption because there are not enough bile acids reaching the intestine to properly emulsify ingested fats. Consequently, fewer fat-soluble vitamins, including vitamin K, are absorbed and a vitamin K deficiency may develop. The deficiency causes impaired carboxylation of vitamin K-dependent coagulation factors, despite normal activity of reducing enzymes. Non-carboxylated factors are produced and enter the blood, where they are known as PIVKA (proteins induced by vitamin K absence). Amounts of vitamin K epoxide would not increase in this case.
3. *Deficiency due to 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.

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with a correction of prolonged PIVKA-sensitive PT values in cats with intestinal or hepatic diseases.[304]

3. Causes of abnormal hemostatic test results
   a. Prolonged PT, PTT: decreased amounts of functional vitamin K-dependent coagulation factors (II, VII, IX, X); PTT alone, PT alone, or both values may be prolonged, depending on the optimization of the assays and, perhaps, the species involved.[383]
   b. Thrombocytopenia: If present, thrombocytopenia probably results mostly from consumption at multiple sites of hemorrhage; when moderate or marked, BMBT prolongations would be expected, especially if the patient is anemic.
   c. Other abnormalities: not widely described, but decreased [FDP], including D-dimer, and increased [fibrinogen] may result from decreased coagulation and consequently decreased fibrinolysis; prolonged TT reported in people correlated with increases in [fibrinogen].[293]
4. Rare hereditary defects in vitamin K-dependent carboxylase produce similar hemostatic abnormalities, but vitamin K is neither deficient nor antagonized in this disorder.[384]

C. Consumptive coagulopathy: coagulopathy resulting from local or disseminated coagulation; caused by consumption of procoagulant blood components and the accompanying generation of FDP. The term *consumption* refers to the removal of active blood components from circulation via the process of coagulation.
   1. Clinical signs vary from subclinical to serious hemorrhage, shock, or signs of multiple organ failures caused by thromboembolism.
   2. May arise from disseminated intravascular coagulation (DIC): Bleeding is common.
      a. DIC is a syndrome of consumptive coagulation occurring throughout the vasculature (disseminated and intravascular) and initiated by massive activation of coagulation and platelets. It may be acute or chronic, compensated or uncompensated.
      b. Coagulation is often initiated by TF release from damaged tissues, neoplastic cells, or endotoxin-stimulated cells or by contact activation caused by massive endothelial cell damage or activation. Many snake venoms initiate the process by activating the common pathway directly.[107,287,385]
      c. When production of procoagulant factors does not keep up with consumption, excessive coagulation can lead to defective coagulation (i.e., consumptive coagulopathy).
   3. May arise from localized consumptive coagulation: Bleeding is rare.[104]
      a. Excessive coagulation in a localized area or organ can produce laboratory signs of consumptive coagulation and, rarely, clinical bleeding. Correction of the localized problem may be curative.
      b. Human examples include aortic aneurysm and large hemangiomas. With these conditions, disseminated intravascular coagulation does not occur, but localized hemostatic abnormalities may lead to a disseminated intravascular coagulopathy. Large cutaneous and splenic hemangiomas and hemangiosarcomas may be examples of localized consumptive coagulation in dogs. However, the changes have usually been interpreted as DIC.
   4. The patterns of laboratory abnormalities vary considerably and are dependent on severity, duration, and underlying or concurrent disorders. Consumptive coagulation can be recognized when the typical laboratory and clinical signs accompany a condition known to trigger the process. Recognition is difficult when the typical pattern is not present.

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5. Causes of abnormal hemostatic test results
   a. Hypofibrinogenemia: Fibrinogen is consumed as it is transformed into fibrin during excessive coagulation. Because fibrinogen is a positive acute phase protein, increased hepatic production may occur in inflammatory states and mask the hypofibrinogenemia typical of consumptive coagulation.
   b. 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.
   c. Prolonged PT, PTT, ACT: hypofibrinogenemia, increased [FDP], and decreased functional coagulation factors
      (1) Once the nonenzymatic coagulation cofactors V and VIII become activated to Factors Va and VIIIa, they are inactivated by protein Ca. Decreased Factors V and VIII concentrations will develop if activation and inactivation occur at a greater rate than production and secretion.
      (2) Coagulation enzymes in the intrinsic and common pathways are bound by ATIII and removed from circulation by hepatocytes. Thrombin also binds thrombomodulin on endothelial cell membranes, and the complex 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.
   d. Decreased ATIII activity: After ATIII binds to activated coagulation enzymes, the complexes are removed from circulation by hepatocytes. If the rate of removal remains greater than the rate of production and release of new ATIII, decreased plasma ATIII activity occurs.
   e. Increased [FDP] and [D-dimer]: With activation of coagulation pathways, there is concurrent activation of fibrinolysis and subsequent plasmin-mediated degradation of fibrin and possibly fibrinogen. This leads to the increased formation of FDP including D-dimers.
   f. Prolonged TT, TT_{Clauss}: hypofibrinogenemia, increased [FDP]
   g. Prolonged BMBT: thrombocytopenia; increased [FDP]

D. Dilutional coagulopathy: Infusion of large volumes of colloid fluids, crystalloid fluids, 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 fibrinogen. This may result in prolonged coagulation times and contribute to hemorrhage, but it is not expected to cause hemorrhage alone.[111,386,387] Compared to saline, high molecular weight colloids (dextrans, hetastarch) may have additional effects on hemostasis, such as causing greater decrements in the amount of plasma vWf:Ag. The effects depend on the fluid infused, the rate of infusion, and the recipient species.

E. Afibrinogenemia/dysfibrinogenemia (rare):[285-288] Without adequate functional fibrinogen, fibrin clots cannot form, thus prolonging PT, PTT, ACT, and TT. Because fibrinogen is the major interplatelet bridge during aggregation, BMBT may be prolonged.

F. Inhibition of coagulation factors[388]
   1. Heparinization: Heparin administration or release from neoplastic mast cells may prolong coagulation times and predispose to hemorrhage (see Fig. 5.5 for mechanism of action).
   2. FDP: FDP contribute to hemorrhage, prolong coagulation times, and decrease platelet function (see Fibrinolysis, II.D.4 above).

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3. Antiphospholipid-protein antibodies (antiphospholipid antibodies):[262] Rarely reported in veterinary medicine,[389] these immunoglobulins react with proteins that bind to anionic phospholipids, e.g., prothrombin, HMWK, Factor XI, protein C, and protein S. They inhibit phospholipid-dependent coagulation tests (e.g., PT, PTT), thus prolonging times if the test plasma is sufficiently devoid of platelets (and therefore phospholipid). Clinical manifestations are thromboembolic events, not hemorrhage. Strict diagnosis requires the following findings:
   a. Prolongation of a phospholipid-dependent coagulation assay
   b. Failure of normal, species-specific, pooled, platelet-poor plasma to correct the prolonged coagulation times when mixed with patient plasma[390]
   c. Neutralization of the inhibitor by increased phospholipid in the test system
   d. Exclusion of other coagulopathies
4. Antibodies to coagulation factors:[391] This problem in human hemophiliac patients receiving multiple transfusions and producing antibodies to the factor they lack also has been reported in transfused dogs with hemophilia A and hemophilia B.[392,393] It results in prolonged coagulation times of the affected pathway or pathways because of antibody-mediated inhibition of the target protein. Normal pooled plasma does not correct the prolonged coagulation times when mixed with patient plasma; prolonged coagulation times reflect an increased propensity to bleed.

THROMBOSIS

I. Thrombosis is the formation of thrombi within the vascular system. Thrombi are *in vivo* "clots" composed of varying amounts of fibrin and blood cells. Thrombi that form under high-flow conditions (arterial) contain more platelets. Thrombi that form under low-flow conditions (venous) contain more erythrocytes and fewer platelets.

II. Thrombi form when the normal balance between prothrombotic factors and antithrombotic factors shifts to favor thrombosis (i.e., a hypercoagulable state, thrombophilia). The specific reasons for this shift are not completely understood for many conditions. The shift may occur with disorders involving:
   A. Endothelial cell activation or damage
   B. Platelet activation
   C. Activation of coagulation
   D. Blood stasis
   E. Inhibition of fibrinolysis
   F. Deficiencies or abnormalities in anticoagulant proteins (e.g., ATIII, protein C, protein S, Factor V) or abnormalities of coagulant proteins (e.g., prothrombin)[394]

III. 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 that may lead to hemorrhage as discussed above. Thrombi may occur in a single area due to local disturbances (e.g., chemical irritants injected into the jugular vein) or at multiple sites due to more systemic abnormalities (e.g., DIC).

IV. Clinical signs are variable and depend on the underlying disease and location of the lesion(s), e.g. dyspnea with pulmonary thromboembolism, hematuria with renal infarction, and rear limb weakness and no femoral pulses with a distal aortic thrombus.[395-399]

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V. Potential hemostatic abnormalities
   A. Increased [D-dimer]: evidence of cross-linked fibrin degradation, and therefore intravascular coagulation
   B. Increased [FDP]: evidence of fibrin degradation, but it could be the result of fibrinogenolysis without intravascular coagulation.
   C. Decreased ATIII activity: evidence of consumption due to intravascular coagulation, but it may result from ATIII loss, in which case it may contribute to intravascular coagulation.
   D. Increased [TAT]: evidence of increased thrombin generation, and therefore intravascular coagulation
   E. Decreased protein C activity: evidence of consumption due to intravascular coagulation, but it may result from other causes, such as vitamin K antagonism or hereditary deficiency, in which case it may contribute to intravascular coagulation.
   F. Thrombocytopenia: evidence of consumption due to intravascular coagulation, but there are many other causes.
   G. Decreased [fibrinogen]: evidence of consumption due to intravascular coagulation, but increased production associated with inflammation may mask shortened fibrinogen survival.

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Chapter 6

BONE MARROW AND LYMPH NODES

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Table 6.1. Abbreviations and symbols in Chapter 6

»	symbol in tables to indicate relatively common disease or condition
CBC	complete blood count
CD	cluster of differentiation
CMMol	chronic myelomonocytic leukemia
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
Epo	erythropoietin
Fe	iron
FeLV	feline leukemia virus
G:E	granulocytic:erythroid
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte/macrophage colony stimulating factor
IL-x	interleukins (x for Arabic numbers)
M:E	myeloid:erythroid
MDS	myelodysplastic syndrome
MDS-EB	myelodysplastic syndrome–excess blasts
MDS-Er	myelodysplastic syndrome–erythroid predominance
MDS-RC	myelodysplastic syndrome–refractory cytopenia
PCR	polymerase chain reaction
WRI	within reference interval

BONE MARROW: MAJOR CONCEPTS AND TERMS

I. Indications for bone marrow examinations include the following:
   A. To pursue the possible causes of unexplained:
      1. Nonregenerative anemia, especially persistent
      2. Neutropenia
      3. Thrombocytopenia
      4. Pancytopenia
      5. Lymphocytosis, especially if atypical lymphocytes are seen in blood films or there is other evidence of lymphoid neoplasia
      6. Thrombocytosis, especially extreme
      7. Erythrocytosis, especially if no evidence of hemoconcentration, splenic contraction, or cardio-pulmonary disease
      8. Mastocytemia
      9. Atypical or immature cells in blood films
      10. Hyperproteinemia, especially if no evidence of hemoconcentration or dehydration
      11. Hypercalcemia
   B. To search for:
      1. Evidence of metastatic neoplasia, especially of lymphocytes and mast cells
      2. Evidence of iron storage, especially when considering iron deficiency
      3. Evidence of specific diseases: histoplasmosis, leishmaniasis, myeloma, storage diseases

II. Different meanings of *myelo-*: The prefix *myelo-* has several meanings and thus a word beginning with *myelo-* must be interpreted in context of the sentence.

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A. *Myel-* or *myelo-*: combining form denoting relationship to bone marrow; however, often used in specific reference to spinal cord or for the nonlymphoid hemic cells (myeloproliferative disease)
B. Myeloid: pertaining to or derived from bone marrow or pertaining to leukocyte precursors (myeloid:erythroid ratio); having appearance of the myelocyte; pertaining to spinal cord
C. Myelocyte: intermediate precursor cell of the granulocytes; any cell of the grey matter of the nervous system
D. Myelitis: inflammation of bone marrow; inflammation of spinal cord
E. Myelogenous: produced in bone marrow

III. Nomenclature for erythroid precursors
A. In veterinary medicine, the nucleated erythroid precursors are named using a *rubri* prefix. The cells within the series are the rubriblast, prorubricyte, basophilic rubricyte, polychromatophilic rubricyte, normochromic rubricyte, and metarubricyte.
B. In human medicine, the nucleated erythroid precursors are named using either erythroblast or normoblast terms. In the normoblast series, the cells are pronormoblast, basophilic normoblast, polychromatophilic normoblast, and orthochromatic normoblast. Similar adjectives are used in the erythroblast series.
C. Accordingly, an increased concentration of nucleated erythroid precursors in the blood is called rubricytosis in veterinary medicine, but it may be called either erythroblastosis or normoblastosis in human medicine.

IV. Methods
A. Complete descriptions of a bone marrow biopsy (collection, fixation, staining, and 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.[1-7]
B. Major features of a bone marrow biopsy
1. Typically, bone marrow samples are collected from sites that are expected to have active hematopoietic tissue.
a. In dogs and cats, such tissue is expected in the iliac crest or in the medullary cavities of proximal femurs or humeri.
b. In horses, such tissue is most accessible in the sternebrae. In cattle, medullary cavities of proximal ribs can be sampled.
2. There are two major methods of collecting bone marrow samples.
a. Aspiration: A Rosenthal or Illinois biopsy needle is inserted into a bone marrow site, stylet is removed, and bone marrow is aspirated into needle and perhaps syringe by negative pressure. Excessive hemodilution is limited by terminating aspiration as soon as blood is seen entering the syringe. The Rosenthal and Illinois needles are straight-shafted needles with a beveled end; nondisposable forms require sterilization and sharpening; disposable forms are available.
b. Core cutting: A Jamshidi biopsy needle is inserted into the cortical bone adjacent to bone marrow, stylet is removed, the needle is advanced about 1 inch into the bone marrow with a twisting motion, the needle is rotated 360° several times and then withdrawn. After the needle is withdrawn, the stylet is used to push the core sample out the top of the needle. The Jamshidi needle (after stylet removal) has a cutting end that is narrower than most of the needle's bore; this promotes retrieval of a cylindrical core of tissue.

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3. There are two major methods of processing bone marrow samples.
   a. Preparation and staining of air-dried films or smears
      (1) It is critical that such samples be processed (sample distributed and fixed onto glass slides) within seconds of collection to reduce clot formation in aspirates or autolytic changes in all samples. A variety of smear, squash, roll, and imprint methods may be used to produce slides that have monolayers of intact cells and foci of bone marrow particles that may be stained and examined.
      (2) 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 DiffQuik). A Prussian blue or Mallory stain may be applied to assess amount of stainable iron in the sample.
      (3) For special microscopic examinations such as cytochemical or immunocytologic methods, slides containing the bone marrow samples may need to be immediately immersed in alcohol or acetone. The slides are then submitted to a hematology laboratory that offers special staining and examination of the bone marrow samples.
   b. Preparation and staining of 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 or Zenker's fixative is preferable, but routine buffered formalin can be used. (Note: if formalin is used, put the air-dried samples in a separate room because formalin fumes can severely alter the staining properties of cells). The fixation procedures vary with the type of fixative fluid that is used.
      (2) The fixed core sample is submitted to a hematology or histology laboratory that can process and stain the sample for microscopic examination. Many core samples require decalcification before the sample can be sectioned and prepared for staining. A variety of stains may be applied to demonstrate the types of cells or other contents of the sample.
4. Examination of bone marrow samples
   a. Gross examination
      (1) Aspirated bone marrow samples will contain peripheral blood cells but the best samples have very little contaminant blood. A representative sample typically contains small pieces of tissue known as bone marrow fragments, bone marrow units, bone marrow grains, bone marrow particles, or bone marrow spicules. The bone marrow fragments that contain adipose tissue will glisten. Bone marrow fragments should not be confused with clotted blood or platelet clumps.
      (2) The appearance of bone marrow core samples will vary from hypercellular red tissue to hypocellular fatty tissue to cortical bone.
   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 a person who is trained for such examinations. A complete examination with differential cell counts and special stains can be a time-consuming process. Frequently, just as much can be learned from a subjective assessment of cell populations by an experienced hematologist. The major aspects of the examination assess the following:

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(1) Cellularity of bone marrow
  (a) Cellularity of bone marrow is best assessed in a core sample, especially in the hypocellular samples. The cellularity of bone marrow fragments reflects the cellularity of the bone marrow. The more particles there are to examine, the better the assessment of the bone marrow cellularity.
  (b) Cellularity of the preparation is not the same as the cellularity of the bone marrow. If bone marrow particles, on average, are < 20%-30% adipose tissue, the bone marrow is hypercellular. If bone marrow particles, on average, are > 70%-80% adipose tissue, the bone marrow is hypocellular. 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) Assessment of hemic precursor populations (see Figs. 3.1 and 6.1)
  (a) Number and structure of megakaryocytes
    (i) The expected number of mature megakaryocytes in a sample varies between species and is highly dependent on the quality of the sample, especially the number of bone marrow fragments in aspirates. If more megakaryocytes are found than are expected, then megakaryocytic hyperplasia is present. If fewer are present, then megakaryocytic hypoplasia is present.
    (ii) The presence of immature forms of the megakaryocytic series (i.e., megakaryoblasts or promegakaryocytes) indicates increased megakaryocytopoiesis, usually megakaryocytic hyperplasia, but rarely megakaryocytic neoplasia.
  (b) G:E ratio
    (i) A G:E ratio is calculated using cytologic samples by differentiating 500 to 1000 consecutive precursors of the granulocytic and erythroid precursors and then dividing the number of granulocytic cells by the number of erythroid cells. All cells of the granulocytic cell lines are included; only the nucleated cells of the erythroid cells are included. Other cells (e.g., lymphocytes, monocytes, macrophages, megakaryocytes, mast cells, and stromal cells) are not included. A G:E ratio is estimated in histologic samples.
    (ii) Most authors seem to use G:E and M:E as synonyms. Others include monocyte precursors in the myeloid cells so that G:E and M:E ratios are not equal. However, these ratios would often be similar because there are usually few recognizable cells of the monocyte series. The M:E versus G:E ratio issue is complicated by the fact that it can be difficult to differentiate monoblasts and promonocytes from granulocytic precursors during routine microscopy.
    (iii) A G:E ratio along with an assessment of bone marrow cellularity and CBC results helps determine if there are hyperplastic or hypoplastic populations of cells.
    (iv) The expected G:E ratios in healthy animals vary between species. Average ratios in dogs, cats, horses, and cattle are reported to be 1.25, 1.63, 0.93; and 0.71, respectively.[1]

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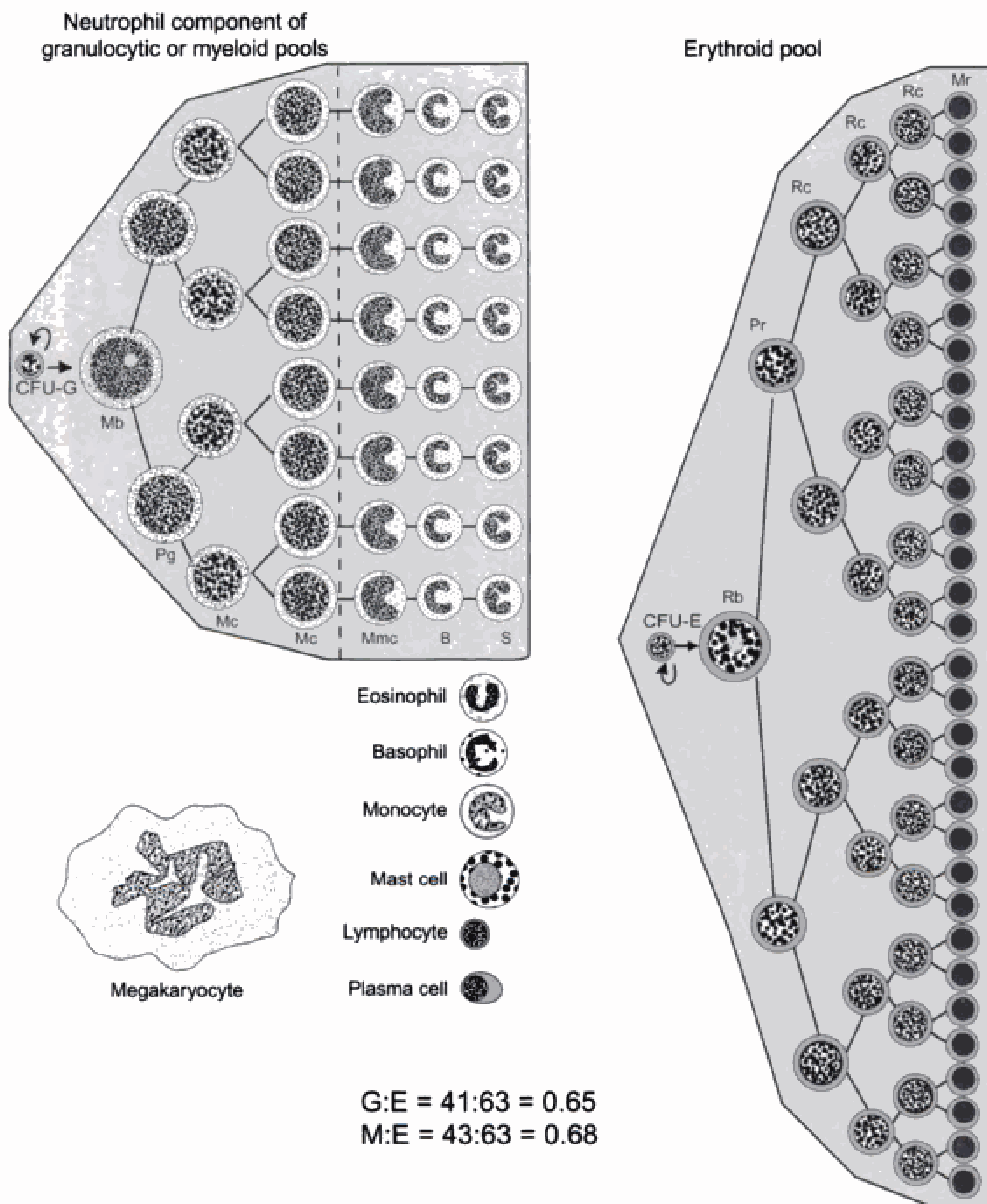

Fig. 6.1. Schematic representation of the hemic cells that may be found in bone marrow of mammals.

The two major pools are cells of the neutrophil series in the granulocytic pool and cells of the erythroid pool. (CFU-G = colony forming unit—granulocyte, CFU-E = colony forming unit—erythroid, Mb = myeloblasts, Pg = progranulocytes, Mc = myelocytes, Mmc = metamyelocytes, B = band neutrophils, S = segmented neutrophils; Rb = rubriblasts, Pr = prorubricytes, Rc = rubricytes, Mr = metarubricytes)

Eosinophils and basophils and their precursors are also components of the granulocytic pool. Monocytes are part of the myeloid pool but not the granulocytic pool. Lymphocytes, mast cells, and megakaryocytes are separate cell lines.

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(c) The granulocytic and erythroid cell lines should contain cells that indicate an orderly proliferation and maturation of the cells.

(i) Because nearly all cells of the erythroid precursors can undergo mitosis, there should be many more of the late stages than the early stages (a pyramidal distribution (see Figs. 4.1 and 6.1). There also should be an orderly and synchronous maturation between nuclei and cytoplasms: as nuclei become smaller and chromatin denser, the size of the cell decreases, the cytoplasmic basophilia decreases, and the eosinophilic staining of hemoglobin increases.

(ii) The granulocytic precursors are divided into two pools (see Figs. 3.2 and 6.1). The early pool (proliferation pool) contains cells capable of mitosis and thus more myelocytes are expected than myeloblasts or progranulocytes. Most of the granulocytes should be segmented neutrophils in the maturation pool (including storage pool). There should be an orderly and synchronous maturation between nuclei and cytoplasms throughout the series.

(d) Most of the lymphocytes in bone marrow of healthy animals are small lymphocytes, but occasional larger forms and plasma cells can be found. These are best seen with cytologic samples.

(3) The relative percentages of other cells (mast cells, macrophages, fibroblasts, osteoclasts, other stromal cells) are determined or estimated. Increased percentages can indicate or suggest certain pathologic processes (see later section on interpretation).

(4) The amount of iron pigment in the sample is estimated. In aspirate and core samples, the iron pigment in hemosiderin will range from yellow to yellow-green to yellow-brown. A more complete assessment of iron storage can be determined if the samples are stained with an iron stain such as Prussian blue or Mallory stains.

c. Histologic versus cytologic examinations

(1) Histologic examination of core samples

(a) Advantages

(i) Can assess tissue architecture (how cells are arranged), necrosis, infiltrative patterns, myelofibrosis

(ii) Better assessment of bone marrow cellularity (more objective)

(iii) Better assessment of megakaryocyte number

(b) Disadvantages

(i) More expensive and greater turnaround time

(ii) More difficult to differentiate cells, especially if sections are not cut thin enough (target ≈ 3 μm)

(2) Cytologic examination of aspirate samples

(a) Advantages

(i) Can critically differentiate and evaluate individual cells and cell populations

(ii) Relatively little processing cost; most of technique expense is in the collection procedure

(iii) Can use for cytochemical staining and have greater potential for immunocytochemistry

(b) Disadvantages

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(i) Cannot assess tissue architecture
(ii) May be difficult to determine if sample is representative (i.e., to differentiate a poor sample from hypocellular bone marrow)

(3) Combining examinations of core and aspirate marrow samples provides the most complete information and the samples can be collected at the same time. Cytologic evaluation of aspirates is almost always indicated. 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 pancytopenia is present) and therefore an aspirate may yield few cells and a pattern that may not be distinct from poor sampling.

d. Post mortem bone marrow samples
(1) Hematopoietic cells undergo autolysis rapidly.
(a) Insides of bones stay warm much longer than most tissues.
(b) Precursors of leukocytes contain many proteolytic and lipolytic enzymes that allow digestion of cells.
(2) Samples must be collected as soon as possible after animal's death so the samples will be adequate for evaluation (guideline: $< 1/2$ hr, especially for cytologic assessment).

BONE MARROW CLASSIFICATIONS

I. Bone marrow hyperplasia: A nonspecific term that indicates an increased number of non-neoplastic hematopoietic cells in bone marrow. Nearly all hyperplastic states result from a stimulus that causes a cell line to proliferate; the stimulus may originate from an increased need for cells or to replace lost cells (Table 6.2.)

A. Erythroid hyperplasia (erythroblastic or normoblastic hyperplasia)

1. Effective erythropoiesis: Epo stimulation of precursor cells leads to a proliferation of erythroid cells, erythroid hyperplasia, and increased release of reticulocytes, nucleated erythrocytes, or erythrocytes into blood.
a. Secondary to hemolytic (Table 4.8) or blood loss disorders (see Fig. 4.6): The mechanisms that lead to erythroid hyperplasia begin immediately after the onset of anemia but the resultant erythroid hyperplasia may not be easily recognized 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 hypoxemia or increased tissue demand for O_2 in tissues promotes Epo release, which causes erythroid hyperplasia and erythrocytosis (see Chap. 4 for disorders).
c. Secondary inappropriate erythrocytotic disorders: Inappropriate Epo production in the absence of hypoxemia causes erythroid hyperplasia and erythrocytosis (see Chap. 4 for disorders).

2. Ineffective erythropoiesis
a. Immune-mediated destruction of nucleated erythroid cells
(1) These disorders are typically considered immune-mediated based on the apparent response to immunosuppressive therapy. In some cases, there may be a concurrent positive Coombs' test or other findings (e.g., thrombocytopenia) that suggests an immune-mediated hemic disorder.
(2) In bone marrow samples, the erythroid series can have the appearance of mat-

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Table 6.2. Disorders and conditions that cause erythroid, granulocytic, and megakaryocytic hyperplasia in marrow

- Erythroid hyperplasia
  - Effective erythropoiesis
    - [a]Secondary to hemolytic or blood loss disorders
    - Secondary appropriate erythrocytotic disorders
      - Right to left shunts, congenital or acquired
      - Chronic pulmonary disease
      - Hyperthyroidism
    - Secondary inappropriate erythrocytotic disorders
      - Renal neoplasms, cysts, or diseases
      - Other neoplasms (hepatoma)
  - Ineffective erythropoiesis
    - [a]Immune-mediated nonregenerative anemia
    - [a]Nutritional: Fe, copper, folate or vitamin B_{12} deficiency
  - Cyclic hematopoiesis of grey collies and in FeLV-infected cats
- Granulocytic hyperplasia
  - Effective granulopoiesis
    - Inflammatory
      - [a]Infections: bacterial, fungal, viral, protozoal
      - [a]Immune hemolytic anemia
      - [a]Necrosis: hemolysis, hemorrhage, infarcts, burns, neoplasia, sterile inflammation
      - Sterile foreign body
    - Other or unknown mechanisms
      - Paraneoplastic neutrophilia
      - Neutrophilia of leukocyte adhesion deficiency
      - G-CSF administration
      - Estrogen toxicosis (early)
      - Cyclic hematopoiesis of grey collies and in FeLV-infected cats
  - Ineffective granulopoiesis
    - Immune neutropenia
    - Suspected in animals: diphenylhydantoin and phenylbutazone toxicosis
    - Chronic idiopathic neutropenia (G-CSF deficiency)
- Megakaryocytic hyperplasia
  - [a]Recovery from thrombocytopenia: withdrawal of myelosuppression or in response to a disorder that causes decreased platelet survival
  - Inflammation: infection, immune-mediated, surgery, trauma

Note: Information about eosinophilic, basophilic, mononuclear-phagocytic, lymphoid, mast cell, and generalized marrow hyperplasia are provided in the text.

uration arrest with erythroid stages prior to the damaged cell being numerous.[17] Macrophages containing any stage from rubriblasts to metarubricytes may be found on cytologic preparations. According to some authors, this type of bone marrow might be seen in the development or resolution of pure red cell aplasia in which there will be marked erythroid hypoplasia or erythroid aplasia.

b. Nutritional deficiencies

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(1) Fe-deficiency anemia: In the Fe-deficient state, anemia develops because erythropoiesis does not produce enough erythrocytes to replace those lost via physiologic or pathologic processes. Erythroid hyperplasia and reticulocytosis may be present, but the release of reticulocytes is not enough to meet the need. As the deficiency progresses, the rubricytes may appear smaller and have less Hgb in their cytoplasms.

(2) There can be a pattern of ineffective erythropoiesis in the rare cases of copper, folate, and cobalamin deficiencies.[1,8]

B. Granulocytic hyperplasia: Unless stated otherwise, granulocytic hyperplasia (also called myeloid hyperplasia) typically is characterized by an increased number of neutrophil precursors. An orderly and complete proliferation and maturation series is expected with hyperplasia, but granulocytic hyperplasia may lack a storage pool or may have a left shift. Other forms of granulocytic hyperplasia are identified as eosinophilic granulocytic hyperplasia and basophilic granulocytic hyperplasia. Because early stages of neutrophil and monocyte precursors are not easily differentiated, a monocytic hyperplasia may or may not be recognized when there is neutrophilic granulocytic hyperplasia.

1. Effective granulopoiesis: Continual stimulation of neutrophil precursors by G-CSF, GM-CSF, or certain interleukins leads to granulocytic hyperplasia, increased release of neutrophils to blood, and typically a neutrophilia (with or without a left shift).
   a. Inflammatory: The granulocytic hyperplasia is typically the result of an inflammatory process that resulted from any of a wide variety of infectious and noninfectious diseases.
   b. Other or unknown mechanisms of granulocytic hyperplasia are listed in Table 6.2 and Chapter 3.
2. Ineffective granulopoiesis: This form of hyperplasia is recognized when there is a neutropenia and a concurrent granulocytic hyperplasia but too few of later stages to complete the pyramidal maturation sequence.
   a. Immune neutropenia: Granulocytic hyperplasia results from persistent differentiation and proliferation of neutrophil precursors in an attempt to replace neutrophils being destroyed before or soon after release from bone marrow. Factors that lead to the generation of anti-neutrophil antibodies typically are not known but might be drug-induced in some cases.
   b. Drug-induced neutropenia: There have been rare reports of an ineffective granulopoiesis associated with anticonvulsant (phenobarbital, primadone) treatments; the mechanism of the neutrophil destruction has not been established but might be immune-mediated.[9]
   c. Chronic idiopathic neutropenia due to G-CSF deficiency: The bone marrow in the rottweiler with this disorder had numerous myeloblasts and progranulocytes, but myelocytes and later neutrophil stages were rare. The defect in maturation resulted from the G-CSF deficiency because G-CSF is needed for terminal neutrophil differentiation.[10]

C. Megakaryocytic hyperplasia: Megakaryocytic hyperplasia occurs when there is persistent stimulation of megakaryocytopoiesis. Stimulation of thrombopoiesis is usually concurrent and secondary to thrombocytopenia, but thrombocytosis may be present and the result of megakaryocytic hyperplasia.

1. Recovery from thrombocytopenia
   a. In response to a thrombocytopenia, stimuli (stem cell factor, thrombopoietin, IL-3, and GM-CSF) promote the proliferation of megakaryocytes to replace the missing platelets.

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b. The thrombocytopenia frequently is due to disorders that cause decreased platelet survival. When thrombocytopenia is due to a myelosuppressive agent or process, there may be a rebound megakaryocytic hyperplasia after removal of the suppression.
2. Mild megakaryocytic hyperplasia may be found concurrent with one of the several forms of reactive or secondary thrombocytosis, including inflammation and nonhemic neoplasia (see Chap. 5).

D. Eosinophilic granulocytic hyperplasia: Eosinophilic granulocytic hyperplasia is characterized by increased numbers of eosinophils and eosinophil precursors in bone marrow.
1. Specific mediators, including IL-5 (eosinophil differentiation factor) and CSF-GM from mast cells, macrophages, and lymphocytes, stimulate eosinopoiesis.
2. Eosinophilic hyperplasia may or may not be recognized in animals with eosinophilia (see Table 3.9).

E. Basophilic granulocytic hyperplasia: Basophilic granulocytic 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 (see Table 3.11).

F. Mononuclear-phagocytic cell hyperplasia: This hyperplastic state is present when there is an increased number of monocytic precursors in the bone marrow. It typically is not seen as a primary bone marrow finding but may be found concurrently with granulocytic hyperplasia.

G. Lymphoid hyperplasia: Lymphoid hyperplasia is seen as a non-neoplastic accumulation of lymphocytes in the bone marrow. In tissue sections of bone marrow, the lymphoid cells may occur in nodules, germinal centers, or as a diffuse population.
1. Because bone marrow is a lymphoid tissue, an inflammatory disease may result in lymphoid hyperplasia in bone marrow samples as well as lymph nodes and spleen. The hyperplastic lymphocytes are typically small lymphocytes, but larger forms and plasma cells may be prominent.
2. A variety of disorders that cause the stimulation of the immune system may cause the lymphoid hyperplasia: canine ehrlichiosis,[3] systemic lupus erythematosus,[11] griseofulvin toxicosis.[12]
3. Small lymphocytes may be up to 20% of the nucleated cells in bone marrow samples from healthy cats, whereas less that 5% (usually < 1%) are expected in dogs, cattle, and horses.
4. When there is granulocytic and erythroid hypoplasia, the lymphocyte population may appear more prominent but may not be a hyperplastic population.

H. Mast cell hyperplasia: Mast cell hyperplasia is seen as a non-neoplastic accumulation of mast cells in the bone marrow.
1. Undifferentiated mast cell precursors originate in bone marrow but are not recognized as a separate cell population in routine bone marrow examinations. Occasional mast cells are found in the bone marrow of healthy dogs, cats, and horses and probably represent a resident population.
2. Bone marrow mast cell hyperplasia has been reported in dogs with aplastic anemia,[13] regenerative anemia, Fe-deficiency anemia, hypoplasia secondary to a Sertoli cell tumor, and lymphoma.[14]
3. Mast cell hyperplasia probably represents a reactive state associated with an inflammatory process. Concurrent plasmacytosis is common.

I. Generalized bone marrow hyperplasia: Generalized bone marrow hyperplasia is present when there is concurrent granulocytic, erythroid, and megakaryocytic hyperplasia. It is

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Table 6.3. Disorders and conditions that cause hypoplastic states in marrow

- Generalized marrow hypoplasia
  - [a]Myelitis: bacterial, fungal, viral, protozoal
  - Toxicosis: bracken fern, cephalosporins, chemotherapeutic agents, estrogen, griseofulvin, phenylbutazone, trichloroethylene, trimethoprim/sulfadiazine
  - Irradiation: whole body or environmental
  - [a]Marrow necrosis due to ischemia, infections, or other states
  - [a]Marrow replacement: neoplasia, myelofibrosis, osteopetrosis
- Selective erythroid hypoplasia
  - [a]Pure red cell aplasia: immune-mediated, after recombinant Epo treatment, possibly parvovirus infection
  - [a]FeLV-induced erythroid hypoplasia
  - Endocrine: hypothyroidism, hypoadrenocorticism, hypoandrogenism
  - Drug-induced: chloramphenicol
- Selective granulocytic hypoplasia
  - Infectious: parvovirus (dogs, cats), FeLV, *Toxoplasma, Ehrlichia* (chronic)
  - Toxic: bracken fern, chemotherapeutic drugs, chloramphenicol (cats), estrogen, griseofulvin, phenylbutazone, diphenylhydantoin
  - Immune neutropenia
- Selective megakaryocytic hypoplasia
  - Toxic: bracken fern poisoning, chemotherapeutic agents, estrogens (exogenous, endogenous), griseofulvin, meclofenamic acid, phenylbutazone, trimethoprim-sulfadiazine (or -sulfonamide)
  - Immune-mediated: amegakaryocytic thrombocytopenia

seen in some cases of immune hemolytic anemia in which there is a concurrent thrombocytopenia and inflammatory neutrophilia. It can be seen in other cases in which there is a need for a proliferation of neutrophils, erythrocytes, and platelets. It should not be confused with polycythemia vera, a neoplastic state that involves the same cell lines but results in erythrocytosis, leukocytosis, and thrombocytosis.

II. Bone marrow hypoplasia: A nonspecific term that indicates a decreased number of hematopoietic cells in the bone marrow. Hypoplastic disorders may be caused by absence of stimulating agents, presence of inhibitors, direct damage to the marrow, or direct damage to individual cell lines (Table 6.3).
   A. Generalized marrow hypoplasia
      1. Aplastic anemia (aplastic pancytopenia) is the pathologic state in which generalized marrow hypoplasia causes nonregenerative anemia, neutropenia, and thrombocytopenia.
      2. Many potential disorders or conditions cause generalized bone marrow hypoplasia but identifying and proving the cause of the disorder is frequently difficult. The insult to the bone marrow may have occurred weeks before the animal became clinically ill.
   B. Selective erythroid hypoplasia: Selective erythroid hypoplasia is a pathologic state in which there is a decreased number of erythroid precursors but the granulocytic and megakaryocytic cell lines are not decreased.
      1. Pure red cell aplasia is a pathologic state in which the selective erythroid hypoplasia is represented by a severe depletion of erythroid precursors that causes development of a normocytic, normochromic, nonregenerative anemia.[15]

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a. Primary pure red cell aplasia is considered an immune-mediated disorder and may be seen concurrently with immune spherocytic hemolytic anemia. The sera of affected dogs can suppress erythropoiesis. Bone marrow examinations reveal marked erythroid hypoplasia. There have been many reported cases in dogs[16,17] and rare reports in cats.[18]
b. Secondary pure red cell aplasia has occurred secondary to treatment with recombinant human Epo in dogs,[19,20] horses,[21,22] and cats. The selective erythroid hypoplasia that occurs in some FeLV-infected cats and possibly in parvovirus-infected or parvovirus-vaccinated dogs is also considered a form of secondary pure red cell aplasia.[15]

2. FeLV-induced erythroid hypoplasia
   a. The Kawakami-Theilen strain of FeLV (A, B, and C subgroups present) causes a severe erythroid hypoplasia in neonatal kittens, but not in weanling or adult cats.[23]
   b. Weanling cats and older cats infected with another strain of FeLV (A and C subgroups present) also can develop erythroid hypoplasia and nonregenerative anemia.[24]
3. Endocrine: The nonregenerative anemias of hypothyroidism, hypoadrenocorticism, and hypoandrogenism are due to erythroid hypoplasia, but the degree of hypoplasia in bone marrow samples can be very mild and not recognized in routine bone marrow examinations.
4. Drugs: Chloramphenicol can induce a transient erythroid hypoplasia in dogs[19,25] and cats.[26] There may be concurrent myeloid and megakaryocytic hypoplasia in cats.

C. Selective granulocytic hypoplasia (agranulocytosis): Selective granulocytic hypoplasia is a pathologic state in which there is a decreased number of granulocyte (neutrophil) precursors but the erythroid and megakaryocytic cell lines are not decreased.
   1. Several infections and drugs have been reported to cause selective granulocytic hypoplasia (Table 6.3). However, since many of them have also been associated with hypoplasia of other cell lines, there may be the appearance of selective granulocytic hypoplasia but damage to other cell lines might not be detected in the same marrow sample. In parvovirus infections, the granulocytic hypoplasia could be due to damage to cells with mitotic potential, depletion of neutrophil pools because of excessive tissue demand, or endotoxin-induced damage to marrow cells.[27,28] In canine monocytic ehrlichiosis, the hypoplasia may be seen in chronic infections, whereas hyperplasia is seen in acute infections.
   2. Animals with immune neutropenias can present with either selective granulocytic hypoplasia or ineffective granulopoiesis.

D. Selective megakaryocytic hypoplasia: Selective megakaryocytic hypoplasia is a pathologic state in which there is a decreased number of megakaryocytes but the erythroid and granulocytic cell lines are not decreased.
   1. Several infections and drugs have been reported to cause selective megakaryocytic hypoplasia (Table 6.3).
   2. Thrombocytopenic animals with immune-mediated damage to megakaryocytes can have megakaryocytic hypoplasia or ineffective thrombopoiesis, depending on the degree or stage of megakaryocyte damage.

III. Myelofibrosis

A. Myelofibrosis is a pathologic state in which there are increased fibrous connective tissue and collagen in the bone marrow. With extensive myelofibrosis, there is a deficiency in hematopoietic tissues and an aplastic anemia may develop.

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B. It is usually considered a reactive or inflammatory fibroplasia and may be the consequence of generalized bone marrow damage. In people and in some animals, it may be secondary to a MDS or myeloproliferative disease in which mediators from the hemic cells stimulate fibrocytic hyperplasia.[29]

IV. Myelitis

A. Myelitis, in the context of hemic disorders, is inflammation of bone marrow. Some people use the word *osteomyelitis* to differentiate "bone marrow" myelitis from "spinal cord" myelitis; others use the word to indicate bone and bone marrow inflammation.

B. There are a several agents that can cause a myelitis.

1. Fungal myelitis (histoplasmosis, blastomycosis)
2. Protozoal myelitis (leishmaniasis)
3. Bacterial myelitis, usually as a result of a bacteremia or a penetrating wound

V. Bone marrow necrosis

A. The term is usually restricted to bone marrows in which necrosis is the primary and predominant abnormality (other pathologic states have necrosis, but the necrosis is not the major pathologic process).

B. Bone marrow necrosis has been associated with toxicants (e.g., in drug studies), DIC, hypoxia, and feline panleukopenia.

C. Myelofibrosis may be the "healing" stage or chronic stage of some bone marrow necrosis disorders.

VI. Myelophthisis

A. Strictly speaking, myelophthisis means marrow (*myelo-*) wasting (*-phthisis*).

B. Common usage: 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, and osteopetrosis. If the disease process has affected most bone marrow sites, animals will develop an anemia, neutropenia, and thrombocytopenia (aplastic anemia).

VII. Bone marrow neoplasia

A. General concepts

1. Neoplasia of bone marrow cells is not as common as many other forms of neoplasia in domestic mammals. The general diagnosis of hemic cell neoplasia 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 very difficult.
2. Terms
   a. *Leukemia:* the presence of neoplastic leukocytes of bone marrow origin in either blood or bone marrow
   b. *Myeloproliferative disease:* As a general term, myeloproliferative disease refers to any neoplastic or leukemic disorder of nonlymphoid hemic cells, including neutrophils, eosinophils, basophils, monocytes, erythrocytes, megakaryocytes, and perhaps mast cells. In human medicine, it is often used more specifically to describe several clonal disorders of pluripotential stem cells characterized by excessive proliferation of differentiated neoplastic cells that have few or no morphologic or functional abnormalities. These are chronic myeloid leukemia, essential

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thrombocythemia, polycythemia vera, idiopathic myelofibrosis, and chronic myelomonocytic leukemia.

c. *Lymphoproliferative disease:* Lymphoproliferative disease refers to a neoplastic proliferation of lymphocytes. If the lymphoid neoplasia primarily involves blood and marrow, it is called a lymphoid leukemia. If the lymphoid neoplasia primarily involves other tissues with little to no blood involvement, it is called a lymphoma.

d. *Acute leukemia:* An acute leukemia typically has many blastic cells (e.g., granulocytic or lymphoid) and duration of the illness is short (unless treated).

e. *Chronic leukemia:* A chronic leukemia typically contains many well-differentiated cells (e.g., neutrophils or lymphocytes) and an animal may live for months after diagnosis even without treatment.

f. *Myelodysplastic syndromes (MDSs):* MDSs are a group of clonal disorders in which there is ineffective hematopoiesis and morphologic evidence in blood or bone marrow of abnormal maturation of hemic cells.[30]

(1) The dysplastic changes that may be observed in these disorders include the following:[31,32]

(a) Dysmyelopoiesis: giant cell size, abundance of azurophilic granules, hypogranular cytoplasms, polyploidy or hypersegmented neutrophil nuclei, Pelger-Huët cell features (hyposegmentation)

(b) Dyserythropoiesis: multiple nuclei, nuclear fragmentation, megaloblastic appearance, excessive cytoplasm relative to the nucleus, abnormal distribution of siderosomes, macrocytosis, unequal nuclear division

(c) Dysmegakaryocytopoiesis and dysthrombopoiesis: nonlobed large nucleus, multiple small nuclei, dwarf or micromegakaryocytes, megaplatelets, abnormal cytoplasmic granularity or vacuolation of platelets

(2) Classifications

(a) In people, the MDSs are classified into five groups but some authors have proposed alternate terms to classify the myelodysplastic states.[33]

(b) A veterinary MDS classification was proposed by an Animal Leukemia Study Group of the American Society for Veterinary Clinical Pathology and divided MDSs into three groups: MDS, MDS-Er, and CMMol.[31] This system was modified by Raskin by dividing MDS into two groups (MDS-RC and MDS-EB) and thus giving four types of MDS in mammals: MDS-RC, MDS-EB, MDS-Er, and CMMol.[32]

(3) Domestic mammals and people with a MDS may develop acute myeloid leukemia and thus MDS has been considered a preleukemic disorder.

(4) Dysplastic changes of hemic cells may not be directly associated with a clonal proliferation and thus may be considered secondary myelodysplasia. Canine disorders in which dysplastic changes were found included lymphoma, myelofibrosis, immune-mediated anemia and thrombocytopenia, multiple myeloma, polycythemia vera (primary erythrocytosis by the criteria of this textbook), and pyometra.[34] (Note: The authors also reported dysplastic features in a Cavalier King Charles spaniel.)

B. Classifications of hemic cell neoplasia

1. The methods of differentiating and the application of the classification criteria for hemic cell neoplasia are beyond the scope of this textbook. Several chapters in *Schalm's Veterinary Hematology,* 5th ed. (Philadelphia: Lippincott Williams & Wilkins, 2000), provide excellent information and examples of the neoplastic states. The major goals

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of the classification schemes are to accurately determine the cell of origin with hopes that such information can be used to reliably provide prognostic information and therapeutic recommendations. Two major problems are present in the classifications schemes: (1) The most simple and inexpensive method (microscopic appearance on a Wright-stained sample) has major limitations and can be very inaccurate. (2) The more complex and expensive methods (immunophenotypic classifications) are not widely available and are expensive, and their use has been limited to date.

2. The major methods of classifying hemic neoplasia are as follows:
   a. Microscopic evaluation of Wright-stained blood films or bone marrow preparations
      (1) The primary value of these evaluations is to detect the presence of neoplastic hemic cells.
      (2) Finding cells that have nuclear or cytoplasmic features that are unique or more common in certain differentiated cell lines is the first step in characterizing the neoplasia, but it can be very difficult to reliably classify the poorly differentiated disorders; i.e., blast cells could be of one of several cell types.
      (3) An accurate classification of the neoplasia is best accomplished by other methods.
   b. Cytochemical stains[35]
      (1) The basic premise behind the use of cytochemical stains is that leukemic cells will contain enzymes or compounds that are common or unique to certain differentiated cells. For example, neutrophils and monocytes contain peroxidase but lymphocytes, erythrocyte precursors, and megakaryocytes do not.
      (2) Cytochemical stains are used primarily for the differentiation of leukemias, and lymphoid cells typically do not stain. However, poorly differentiated myeloid precursors may not contain enough analyte for a positive reaction.
      (3) The use of cytochemical stains for differentiation of leukemias is limited to a few special hematology laboratories and is of most value when a panel of stains is used, e.g., peroxidase, Sudan black B, leukocyte alkaline phosphatase, naphthyl acetate esterase, naphthyl butyrate esterase, chloracetate esterase, periodic acid-Schiff.
   c. Immunophenotyping or immunocytochemistry[36]
      (1) The use of monoclonal antibodies has allowed the identification of surface antigens on a variety of cell types. If a group or cluster of monoclonal antibodies recognizes the same antigen, the antigen is assigned a cluster of differentiation (CD) number.
      (2) The CD antigens are best characterized on human and mouse cells, but many of the same CD antigens are found on domestic mammal cells. For example, CD3 is a marker for T lymphocytes and CD79 is a marker for B lymphocytes. Other CD antigens are markers of T helper cells, T cytotoxic cells, and the unique cells within myeloproliferative diseases.
   d. Polymerase chain reactions (PCR) for assessment of clonality[37]
      (1) With only rare exceptions, neoplasia of lymphoid cells is an uncontrolled proliferation of one clone of lymphocytes.
      (2) Special PCR assays that evaluate the DNA sequences of lymphocytes are being used to establish that a proliferation of lymphocytes is either a monoclonal (neoplastic) or a polyclonal (hyperplastic) proliferation.
3. Classification schemes (Table 6.4)

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Table 6.4. Classifications of hemic cell neoplasia involving blood or marrow

Classification	Cell of origin
Lymphoproliferative disorders	Lymphocyte
»Acute lymphocytic leukemia	B or T lymphocyte
Chronic lymphocytic leukemia	B or T lymphocyte
»Lymphoma	B or T lymphocyte
Myeloma	B lymphocyte
Large granular lymphoma or lymphocytic leukemia	T or NK lymphocyte
Myeloproliferative disorders	Nonlymphoid hemic cell
Acute myeloid leukemias[a]	Nonlymphoid hemic cell
Acute myeloblastic without maturation (M0)[b]	Granulocyte or monocyte
Acute myeloblastic with minimal maturation (M1)	Granulocyte or monocyte
Acute myeloblastic with maturation (M2)	Granulocyte or monocyte
Promyelocytic leukemia (M3)	Granulocyte
Myelomonocytic leukemia (M4)	Granulocyte and monocyte
Monocytic leukemia (M5)	Monocyte
Erythroleukemia (M6)	Erythrocyte and granulocyte
Erythroleukemia with erythroid predominance (M6-Er)	Erythrocyte and granulocyte
Megakaryocytic leukemia (M7)	Megakaryocyte
Acute undifferentiated leukemia	Unknown hemic stem cell
Chronic myeloproliferative disorders	Myeloid cell
Chronic myeloid leukemia	Neutrophil
Chronic myelomonocytic leukemia (CMMol)[c]	Neutrophil and monocyte
Eosinophilic leukemia	Eosinophil
Basophilic leukemia	Basophil
Primary erythrocytosis	Erythrocyte
Polycythemia vera	Erythrocyte, neutrophil, and megakaryocyte
Thrombocythemia	Megakaryocyte
Myelodysplastic syndrome (MDS)[d]	Nonlymphoid hemic cell
MDS-erythroid predominance (MDS-Er)	Nonlymphoid hemic cell
MDS-refractory cytopenia (MDS-RC)	Nonlymphoid hemic cell
MDS-excess blasts (MDS-EB)	Nonlymphoid hemic cell
Chronic myelomonocytic leukemia (CMMol)[c]	Neutrophil and monocyte
Mast cell neoplasia	Mast cell
»Metastatic mast cell neoplasms (cutaneous or visceral)	Mast cell
Mast cell leukemia	Mast cell

[a] Nomenclature of this classification system was recommended by an Animal Leukemia Study Group of the American Society for Veterinary Clinical Pathology. (Jain NC, Blue JT, Grindem CB, Harvey JW, Kociba GJ, Krehbiel JD, Latimer KS, Raskin RE, Thrall MA, Zinkl JG. 1991. Proposed criteria for classification of acute myeloid leukemia in dogs and cats. Vet Clin Pathol 20:63-82.)

[b] "M" abbreviation system is used in the French-American-British (FAB) classification.

[c] CMMol may be difficult to classify: some may meet criteria for a myeloproliferative disease, others may meet criteria for a MDS.

[d] Proposed classification for MDSs in domestic mammals. (Raskin RE. 1996. Myelopoiesis and myeloproliferative disorders. Vet Clin North Am Small Anim Pract 26:1023-1042.)

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INTERPRETING RESULTS OF BONE MARROW EXAMINATIONS

I. Interpretation of the results of bone marrow examinations is easier *if* you are using the bone marrow examination to answer specific questions.
   A. Why does an animal have a nonregenerative anemia?
   B. Why does an animal have a persistent neutropenia?
   C. Why does an animal have a thrombocytopenia?
   D. Is there a neoplastic process in the bone marrow?

II. Critical interpretation of most bone marrow samples is only possible when there are CBC results for the day the bone marrow was collected.
   A. An animal may have a nonregenerative anemia one day but a regenerative anemia the next day (or the reverse).
   B. An animal may have a neutropenia one day but a normal to increased neutrophil concentration the next day (or the reverse).
   C. An animal may have a thrombocytopenia one day but a normal to increased platelet concentration the next day (or the reverse).
   D. Correlation of the results of a CBC and bone marrow examination
      1. Results of a bone marrow biopsy are best interpreted with current CBC results and other clinical information.
      2. Examples of the correlation of results for five dogs are shown in Fig. 6.2.

LYMPH NODES: MAJOR CONCEPTS AND TERMS

I. Terms and conditions
   A. A lymphadenopathy is a pathologic state involving a lymph node. In clinical diseases, the lymphadenopathies typically cause enlarged lymph nodes (i.e., lymphadenomegaly).

Fig. 6.2. Schematic examples of the interpretation of CBC and marrow biopsy results.

Reference intervals and other expected results for healthy dogs are provided in the right column. The number of marrow megakaryocytes is represented by the number and size of schematic megakaryocytes. Granulocytic and erythroid pools are represented by pool diagrams that are miniatures of those shown in Chapters 3 and 4. G:E ratios were calculated from the number of cells illustrated in the granulocytic and erythroid pools.

- *Dog 1:* Selective erythroid hypoplasia has produced a nonregenerative anemia, decreased fragment cellularity, and an increased G:E ratio.
- *Dog 2:* Erythroid hyperplasia has produced a reticulocytosis, increased fragment cellularity, and a decreased G:E ratio. The regenerative anemia is probably due to blood loss or hemolysis.
- *Dog 3:* Generalized marrow hypoplasia has produced an aplastic anemia (aplastic pancytopenia), decreased fragment cellularity, and a G:E ratio WRI.
- *Dog 4:* Generalized marrow hyperplasia has produced a reticulocytosis, neutrophilia, and increased fragment cellularity. The regenerative anemia is probably due to blood loss or hemolysis, the neutrophilia is due to an inflammatory process, and the thrombocytopenia is due to decreased platelet survival. This dog's inflammatory neutrophilia could be associated with an immune-mediated anemia and immune-mediated thrombocytopenia.
- *Dog 5:* Granulocytic neoplasia has produced myelophthisis and resulted in a nonregenerative anemia due to erythroid hypoplasia, thrombocytopenia due to megakaryocytic hypoplasia, and neutropenia due to defective neutropoiesis (neoplasia).

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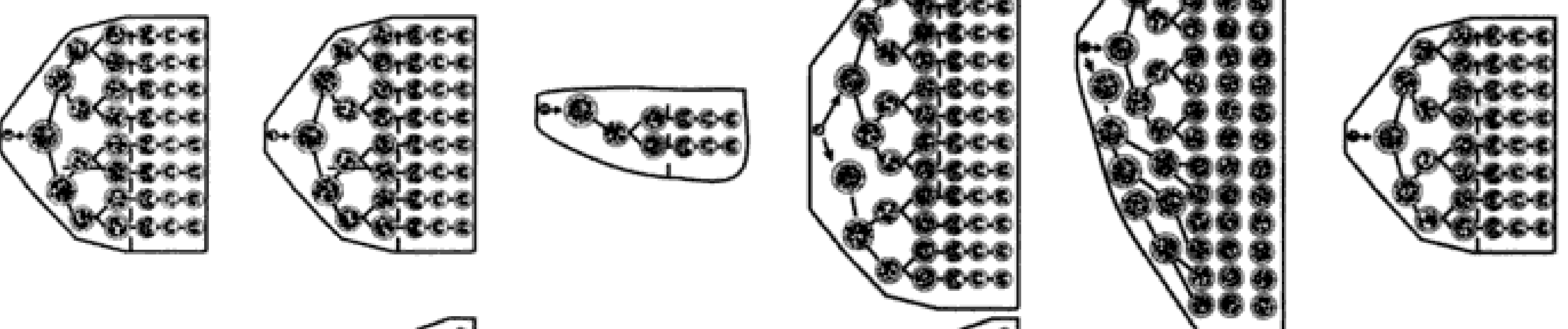
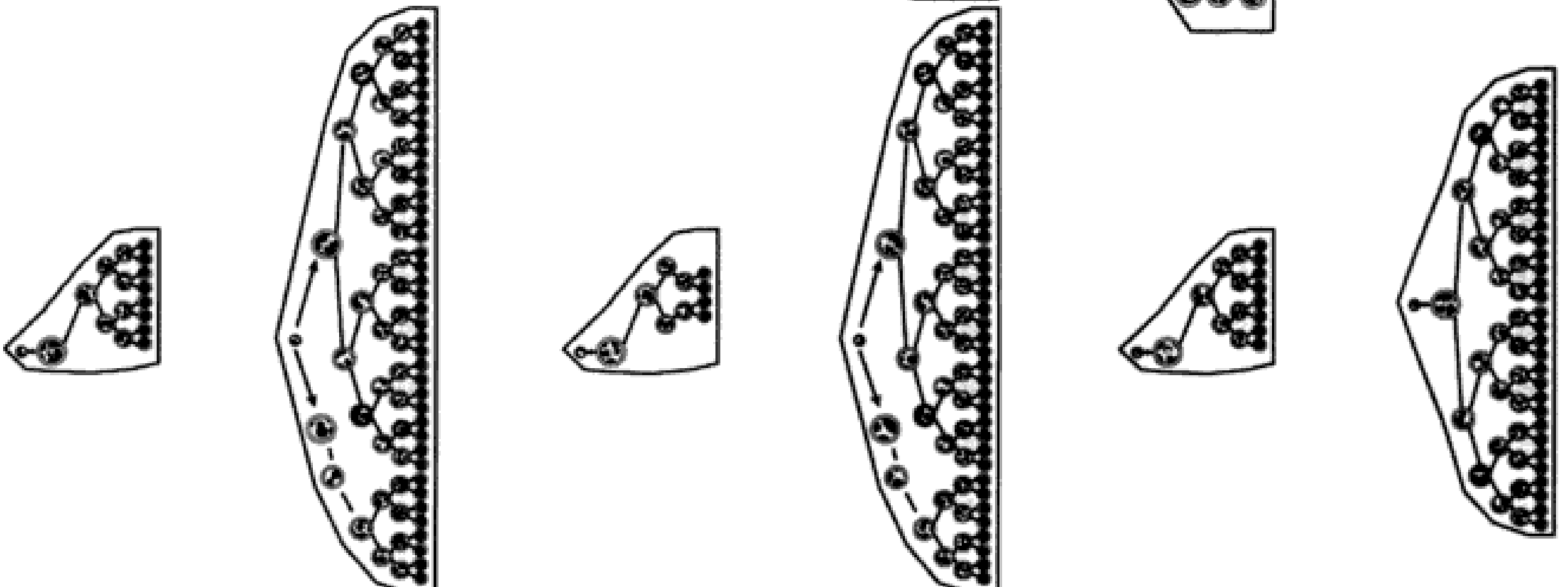
CBC results	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Ref. Int.
Hct (%)	10	10	10	15	10	37–55
Reticulocytes (#/μL)	10,000	200,000	10,000	200,000	10,000	0–80,000
Neutrophils (#/μL)	8,000	8,000	500	30,000	100,000	3,000–12,000
Platelets (#/μL)	300,000	300,000	20,000	20,000	20,000	200,000–500,000

Bone marrow biopsy results						
Fragment cellularity (%)	25	80	10	85	90	30–70
G:E	2.4	0.5	1.0	0.7	3.5	0.9–1.8

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Frequently, but inaccurately, *lymphadenopathy* and *enlarged lymph node* are considered synonyms.

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 major disorders or conditions that cause enlarged lymph nodes are:
   1. Hyperplasia of lymphoid cells
   2. Inflammation of lymph nodes
   3. Neoplasia of lymphoid cells
   4. Neoplasia of nonlymphoid cells (typically metastatic neoplasia)

II. Methods

A. Complete descriptions 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 of a lymph node biopsy are described in several sources.[5,38-40]

B. Major features of a lymph node biopsy
   1. Sample collection
      a. Typically, lymph node samples are collected from enlarged peripheral lymph nodes: mandibular, prescapular, axillary, popliteal, inguinal, and occasionally facial. 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. Methods of collecting cells from the lymph node include fine-needle aspiration, cores from Tru-cut needles, wedge-incision samples, and lymph node excision. After the sample is collected, it may be prepared for cytologic analysis, tissue sectioning, culturing, or other procedures.
   2. Sample processing
      a. The major goal of the cytoprepatory methods is to produce areas on the slide that contain a monolayer of intact lymphoid cells. Some regions of such slides will be too thick and other areas will have marked cell lysis. The air-dried slides should not be exposed to formalin fumes because the gas can severely alter the staining properties of the cells.
      b. Thin slices of excised samples should be placed in fixative for paraffin-embedded sectioning. Formalin is used most often, but B5 fixation provides better fixation for examination of cell detail.
   3. Cytologic examination of lymph node samples
      a. A cytologic examination of lymph node samples involves the differentiation and characterization of nucleated cells and the identification of organisms or other noncellular structures (e.g., hemosiderin).
         (1) The types of lymphocytes are determined by their nuclear diameters, chromatin patterns, presence of enlarged nucleoli, and cytoplasmic features. In health, most lymphocytes should be small lymphocytes with clumped chromatin patterns and scant cytoplasms.
         (2) Other cells are identified by their unique features. Nonlymphoid cells include neutrophils, macrophages, eosinophils, mast cells, metastatic neoplastic cells, and hematopoietic precursors.
      b. Such examinations are best completed on a quality stained sample with a quality microscope by a person who is trained for such examinations.

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4. Methods involved in the histologic examination of lymph node samples are beyond the scope of this textbook. Such examinations should be done by veterinary pathologists.

C. Cells in lymph nodes of healthy mammals
   1. Lymph nodes from healthy mammals are not commonly evaluated. However, the microscopist should have a clear image of what should be seen in normal lymph nodes so that abnormal cell populations or other significant findings will be recognized.
   2. The expected cell populations in lymph nodes vary with the location of the lymph node. Mandibular lymph nodes in healthy mammals typically have higher percentages of resident macrophages, plasma cells, and neutrophils than do other peripheral lymph nodes. Mesenteric lymph nodes also have more macrophages and neutrophils.

LYMPH NODE CLASSIFICATIONS

I. Hyperplastic lymph node
   A. Lymph node hyperplasia is characterized by increased numbers of lymphocytes: B cells, T cells, or both. The proportions of different types of lymphocytes may appear normal, in which case hyperplasia is suggested by normal cell populations in association with lymphadenomegaly. There may be increases in lymphoblasts 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 lymphoblasts. The percentage of lymphocytes that are lymphoblasts is expected to be less than 50% and is usually less than 10%. An increase in plasma cells indicates B-cell stimulation.
   B. The causes of a reactive lymph node are essentially the same as those for lymph node hyperplasia.

III. Lymphadenitis
   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).
   B. The cause of the inflammatory state may be within the lymph node or, more commonly, in the node's drainage field. For example, an allergic dermatitis may result in an eosinophilic lymphadenitis, or a lymph node draining a necrotic hemorrhagic lesion may have many macrophages containing cell debris and Fe pigments.
   C. There may or may not be a concurrent inflammatory leukocytosis in mammals with a lymphadenitis.

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D. Organisms such as pyogenic bacteria, *Mycobacterium* sp., *Histoplasma* sp., *Blastomyces* sp., *Leishmania* sp., *Prototheca* sp., and *Neorickettsia* sp. may be present.

E. Lymphadenitis is often associated with reactive changes, and the term *reactive lymphadenitis* is sometimes used to reflect both changes.

IV. Lymphoid neoplasia (lymphoma, lymphosarcoma)

A. Lymphoma is characterized by the presence of a population of neoplastic lymphocytes. Depending on the appearance of the cells, lymphoma can be an easy or difficult diagnosis.

B. Over the past 30 years, there have been several classifications systems for lymphomas based on tissue patterns, cell sizes, nuclear shapes, nucleolar sizes, and cytoplasmic granulation. Most classifications were formulated for the classification of human lymphomas and their application to other mammalian lymphomas has been inconsistent.

1. Rappaport classification is based primarily on sizes of the lymphocytes and patterns of cell growth (nodular and diffuse).
2. Kiel classification is based on lymphocyte sizes, nuclear and cytoplasmic features, and separates lymphomas into B-cell and T-cell neoplasms.
3. The Lukes-Collins classification is based on the sizes and shapes of the nuclei (e.g., small, large, cleaved, noncleaved, convoluted), cellular features (small, immunoblastic, plasmacytoid, histiocytic), and separates lymphomas into B-cell and T-cell neoplasms.
4. The Working Formulation system uses growth patterns (follicular, diffuse), nuclear outline (cleaved, noncleaved, convoluted), and cell sizes to classify the lymphomas into three grades (low, intermediate, high).
5. The revised European/American Lymphoma (REAL) system divides lymphomas into B-cell or T-cell lymphomas based on immunophenotyping using CD antigens, cell structure, genetic features, and clinical features. A group of specialists organized by the World Health Organization (WHO) modified the REAL system by incorporating aspects of the prior classifications (B cell, T cell, NK cell).[41]

C. As stated in the bone marrow section, there are many ways of evaluating lymphomas. The microscopic evaluation of stained cells may be the easiest and have the most clinical application, but it has major limitations. When cytologic preparations consist of monotonous populations of large lymphoblasts with prominent nucleoli, the diagnosis of lymphoma is clear. However, histologic examination may be required for small-cell lymphomas or when there is a mixture of lymphocyte types due to a nondiffuse form or a recent onset. A lymph node is unlikely to be reactive and likely to be lymphomatous if > 50% of the lymphocytes are lymphoblasts.

D. Immunophenotyping and PCR analysis can provide a more exacting lymphoma classification, but use of the techniques is currently limited by expense, availability, reagents, and lack of need.

V. Nonlymphoid neoplasia (typically metastatic)

A. Lymph nodes can be enlarged because of the growth of nonlymphoid neoplastic cells in the node. Metastatic cells can also be found during biopsies of lymph nodes that do not appear enlarged.

B. Many neoplasms have the potential to spread to regional lymph nodes. Those seen more frequently in the peripheral lymph nodes included squamous cell carcinoma, mammary carcinoma or adenocarcinoma, melanoma, and mast cell neoplasia.

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VI. Other findings
   A. Edema may be suggested by the loose arrangement of cells that suggests dispersion of cells by fluid.
   B. The presence of nonlymphoid hemic precursors (rubricytes, megakaryocytes, myeloid cells) suggests extramedullary hematopoiesis. Extramedullary hematopoiesis may occur when there is extensive damage to bone marrow and stimuli promote proliferation of hemic precursors in nonmarrow sites.
   C. The presence of erythrocytes in the sample indicates hemorrhage. If only erythrocytes are found, it may be difficult to differentiate pathologic hemorrhage from hemorrhage due to sampling. The presence of erythrophages and siderophages support the conclusion of pathologic hemorrhage either within the lymph node or its drainage field.
   D. A diagnosis of metastatic melanoma should be considered anytime melanin pigment is found. However, melanin pigment can be found in macrophages (melanophages) when the lymph node's drainage field contains a melanoma or necrosis or inflammation of pigmented tissue.

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Chapter 7

PROTEINS

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Table 7.1. Abbreviations and symbols in Chapter 7

*	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
ADH	antidiuretic hormone
Alb	albumin
BCG	bromcresol green
BCP	bromcresol purple
C3a	complement factor 3a
CBC	complete blood count
DIC	disseminated intravascular coagulation
ECF	extracellular fluid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FDP	fibrin or fibrinogen degradation product
FPT	failure of passive transfer
Hct	hematocrit
HDL	high density lipoprotein
Hgb	hemoglobin
Hpt	haptoglobin
IEP	immuno-electrophoresis
IgA	immunoglobulin A
IgG	immunoglobulin G
IgG(T)	immunoglobulin G, subtype T (T is for tetanus)
IgM	immunoglobulin M
LDL	low density lipoprotein
M_r	relative molecular weight
Na_2SO_3	sodium sulfite
NH_4^+	ammonium
PLE	protein-losing enteropathy
PLN	protein-losing nephropathy
PP:F	plasma protein to fibrinogen ratio (original)
pTP_{ref}	plasma total protein by refractometry
RID	radioimmunodiffusion
SI	Système International d'Unités
SIADH	syndrome of inappropriate ADH secretion
SPE	serum protein electrophoresis
SRID	single radial immunodiffusion
sTP_{ref}	serum total protein by refractometry
Tf	transferrin
TIBC	total iron binding capacity
TP	total protein
TP_{ref}	total protein by refractometry
$(TP:Fib)_p$	total protein to fibrinogen ratio in plasma
WRI	within reference interval
$ZnSO_4$	zinc sulfate

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GENERAL CONCEPTS

I. Physiologic processes

A. Proteins are polypeptide chains of amino acids. 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; glycoproteins contain proteins and polysaccharides (sugar).

B. Plasma contains fibrinogen and other clotting factors. Serum is the fluid remaining after blood clots and does not contain fibrinogen. Most plasma proteins are synthesized by hepatocytes; the major exceptions are the immunoglobulins that are produced by B-lymphocytes and plasma cells.

II. Protein disorders

A. Protein dyscrasia: a condition where there is an abnormal protein (abnormal structure)

B. Dysproteinemia: the presence of normal protein at abnormal concentration or abnormal protein in blood

1. Selective or nonselective dysproteinemias
   a. Nonselective hyperproteinemia: All protein concentrations are increased (panhyperproteinemia). It results from hemoconcentration.
   b. Selective hyperproteinemia: Some protein concentrations are increased more than others. Typically, it results from inflammation or B-lymphocyte neoplasia.
   c. Nonselective hypoproteinemia: All protein concentrations are decreased (panhypoproteinemia). It results from a proportional loss of proteins or proportional decrease in synthesis.
   d. Selective hypoproteinemia: Some protein concentrations are decreased more than others. It results from selective loss (typically small proteins selectively lost) or from selectively decreased synthesis of one or more proteins.
2. To determine if the dysproteinemia is selective or nonselective, serum protein electrophoresis 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 a 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 not decreased proportionately, then there is a selective hypoproteinemia.
   c. The same concepts apply to hyperproteinemia evaluations.

ANALYTICAL PRINCIPLES

I. Methods of measuring [TP]

A. Refractometry for measuring [TP] (plasma or serum)

1. Principle: The degree of light refraction in an aqueous solution is proportional to the quantity of solids in solution. Because most solids in plasma are proteins, the degree of light refraction is highly dependent on protein concentration.
2. The refractometer's TP scale is calibrated with the assumption that changes in refractive index reflect changes in protein concentration alone. A temperature-compensated

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refractometer is recommended over a non-temperature-compensated refractometer for two reasons.

a. It does not require daily adjustments based on ambient temperatures.
b. 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 about 0.7 g/dL at 85°C (Leica TS400 Total Solids Refractometer literature).

3. Interferences
   a. Because refractive index of a solution is dependent on concentration of solids in the sample, high concentrations of a variety of substances (e.g., glucose, urea, Na^+, Cl^-) could increase the refractive index and thus the TP reading. The TP reading is reported to be falsely increased by 0.6 g/dL if plasma glucose concentration is approximately 700 mg/dL (or 0.7 g/dL) or urea nitrogen concentration is approximately 300 mg/dL.[1]
   b. Gross lipemia will increase the refractive index and thus falsely increase the TP reading.
   c. Hemolysis resulting in a plasma [Hgb] of 0.5 g/dL did not interfere with refractive index values but made reading of the dividing line in the refractometer more difficult.[2]
   d. Bilirubin concentrations at 0.4 mg/dL did not interfere with refractive index values;[2] however, icterus is commonly listed as a cause of falsely increased values in clinical chemistry textbooks. Perhaps interference occurs at higher concentrations.
4. Unit conversion: g/dL × 10 = g/L (SI unit, nearest 1 g/L)[3]
5. Comments
   a. Determination of [pTP_{ref}] is part of CBC tests in many veterinary laboratories because it is a simple, quick, and inexpensive method for detection of hyperproteinemia and hypoproteinemia.
   b. Most refractometers are calibrated for the normal proteins in human plasma. The calibration scale will vary between species because of the different composition of plasma proteins,[2] but the difference is typically considered clinically insignificant.
   c. [TP] in serum may also be estimated with a refractometer; serum concentration will be lower than plasma [TP] because of the absence of fibrinogen in serum. However, there are other factors that result in differences between plasma and serum [TP] even if measured by the same method.[4]
      (1) H_2O diffuses from erythrocytes during clotting and thus lowers serum [TP]. As this change is rarely described, it may cause only minor changes.
      (2) Some anticoagulants (e.g., citrate, oxalate, fluoride) cause H_2O to diffuse from erythrocytes, but heparin (if used in appropriate amounts) does not. The solutes of the anticoagulant will add to the refractive index.
   d. As light refraction is a physical property, [TP] determined via refractometry may not be the same as determined by biuret reaction. In fact, it is frequently mildly different ($\leq$ 0.3 g/dL) but occasionally different by as much as 2.0 g/dL in samples that are not hemolyzed, icteric, or lipemic.

B. Biuret reaction for measuring [TP] (serum)
   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

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pure polypeptides that contain the same amount of nitrogen by weight. Therefore, [TP] determinations are not completely accurate.

2. Interferences: In some assays, hemolysis may cause a positive interference (e.g., Hgb at 400 mg/dL will produce a 12% bias). Dextran may also cause a positive interference. Small peptides may react but contribute very little to total color change; [NH_4^+] is too low to interfere.
3. Unit conversion: g/dL × 10 = g/L (SI unit, nearest 1 g/L)[3]
4. Comment: The biuret reaction is the most common spectrophotometric method of measuring serum [TP].

II. Methods for measuring albumin concentration

A. BCG dye binding reaction (serum)

1. Principle: BCG preferentially binds to albumin and produces a color complex; the quantity of BCG-Alb complex is proportional to the albumin concentration.
2. Interferences
   a. The binding of BCG to globulins will result in a falsely elevated albumin concentration. The nonalbumin binding may lead to significant errors when true serum albumin concentrations are very low (< 1 g/dL) compared to interfering globulins (e.g., α_2-macroglobulin) concentrations.
   b. In some assays, Hgb at 0.4 g/dL will cause a positive 24% bias; triglyceride at 0.8 g/dL will give a negative interference of about 0.2 g/dL.
3. Unit conversion: g/dL × 10 = g/L (SI unit, nearest 1 g/L)[3]
4. Comment: BCG dye binding is the most common spectrophotometric method of measuring serum albumin concentration.

B. BCP dye binding reaction (serum): BCP binding is used in some human medical laboratories but BCP does not reliably bind with all mammalian albumin molecules. BCP assays may give falsely low (sometimes markedly low) results in some domestic species (e.g., dogs).

C. HABA (2-[4′-hydroxyazobenzene]-benzoic acid) dye binding reactions (serum): unreliable in domestic mammal serum[5]

D. Protein electrophoresis (see V below in this section)

III. Methods of measuring or determining total globulin concentration

A. Total globulin concentration is determined by subtraction (serum).

1. Principle: All proteins in serum other than albumin are globulins.
2. Globulin concentration = TP concentration - albumin concentration.

B. Protein electrophoresis (see V below in this section)

C. Unit conversion: g/dL × 10 = g/L (SI unit, nearest 1 g/L)[3]

D. Comments

1. Globulin concentration will be only as accurate as the measured TP and albumin concentrations.
2. Globulin concentration represents the total concentration of all serum proteins other than albumin (more than 1000 proteins; e.g., haptoglobin, transferrin, α_2-macroglobulin, lipoproteins, and immunoglobulins).

IV. Fibrinogen concentration in plasma

A. Heat precipitant method

1. Principle: The difference in [TP_{ref}] in a sample before and after removal of fibrinogen

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via heat precipitation (56°–58°C) and centrifugation estimates the fibrinogen concentration.

2. Unit conversion: mg/dL × 0.01 = g/L (SI unit, nearest 0.1 g/L)[3]
3. Comments
   a. A semiquantitative technique used to screen for hyperfibrinogenemia (mostly bovine and equine plasma)
   b. The method's analytical sensitivity is inadequate to document hypofibrinogenemia. As each refractometric reading is at best only accurate to the nearest 0.1 g/dL, the calculated fibrinogen value should be considered to be at best within 0.2 g/dL of the true value.
4. Outline of heat precipitant method
   a. Fill two microhematocrit tubes (at least 3/4 full) with EDTA-anticoagulated blood. Spin tubes in a microhematocrit centrifuge (as for Hct) for 5 min.
      (1) First tube: Determine $[pTP_{ref}]$ to the nearest 0.1 g/dL.
      (2) Second tube: Place in 56°–58°C H_2O bath for 3 min, then spin in microhematocrit centrifuge as before (to pack the precipitated fibrinogen), then determine the [TP] via refractometer to the nearest 0.1 g/dL.
   b. Calculate fibrinogen concentration
      (1) Estimated fibrinogen concentration = $[pTP_{ref}]$ of first tube - $[TP_{ref}]$ of second tube.
      (2) Example: 7.0 g/dL - 6.7 g/dL = 0.3 g/dL

B. Thrombin time (thrombin clotting time)
   1. Principle: Thrombin time is the time required for fibrin formation after the addition of thrombin to citrated plasma. Thrombin time is primarily dependent on fibrinogen concentration; the lower the fibrinogen concentration, the longer the thrombin time.
   2. See Chapter 5 for details pertaining to thrombin time and the Clauss modification of thrombin time.

C. Some people have attempted to calculate a fibrinogen concentration by determining the difference between the $[pTP_{ref}]$ of EDTA-plasma and a $[TP_{ref}]$ from a serum sample. Such a method is not recommended because of nonfibrinogen factors that cause differences between plasma and serum total protein concentrations (see Analytical Principles, I.A.5 above). Also, errors can arise when two different samples are collected and compared.

V. SPE for determining protein fractions

A. Principles
   1. Serum proteins separate into four to six major groups of one or more bands based on their ability to migrate through cellulose acetate or agarose in an electrical field. The degree of migration towards the anode (positively charged terminal) is based on electrical charge and a protein's mass and shape. In domestic mammal sera, albumin migrates the farthest because it is small and very anionic. Smaller proteins may not migrate as far because they lack the marked negative charge. Other globulins (e.g., α_2-macroglobulin) are very large but negative charges cause an anodal migration. Some immunoglobulins are large and cationic and thus migrate towards the cathode or do not migrate.
   2. Major variations
      a. The same protein groups in each animal species have slight to moderate differences in migrations.
      b. Electrophoresis using cellulose acetate separates the proteins into 5 to 9 protein

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bands, whereas using agarose separates the proteins into 10 to 15 protein bands. A protein band may represent one protein or several proteins that have migrated the same distance.

c. Protein bands that represent globulin proteins are grouped into electrophoretic regions. Via routine cellulose acetate methods, the common groups for domestic animals are as follows (note: a protein concentration above 0.1 g/dL is needed before it can be detected by this method).
   (1) In most dog, cat, and horse sera, five globulin regions can be seen: α_1, α_2, β_1, β_2, and γ.
   (2) In most cattle sera, only three globulin regions are seen: α, β, and γ.

B. Calculating concentrations of the protein fractions
   1. The protein concentration of an electrophoretic group is the product of the [TP] (preferably from biuret reaction) and the percentage of TP occupied by a region. When a stained cellulose acetate strip is scanned with a densitometer, stained proteins cause less light to be transmitted through the strip to a detector. The decreased transmittance is recorded as a deflection on a densitometer scan or tracing. After the cellulose acetate strip is scanned, the resulting curve represents the relative quantities of proteins (Fig. 7.1). The area under the curve represents the total quantity of stained protein.
   2. Densitometers are calibrated so that complete transmittance through the acetate strip results in no deflection of the needle (zero response). In addition, densitometers may be calibrated so that the most blockage of light caused by the darkest protein band results in nearly 100% deflection of the needle (maximum response). The darkest band (or the maximal response) is normally the albumin band but can be found in the globulin fractions.
   3. The percentage of the total area under the curve for each region is calculated to determine the percentage of [TP] represented by each electrophoretic region. Then, the percentages are multiplied by the [TP] to determine the approximate protein concentrations in each electrophoretic region. For example, if the [TP] is 6.0 g/dL and electrophoresis results indicate that 50% of the stained protein is in the albumin band, then the albumin concentration is calculated to be 3.0 g/dL.
   4. Cellulose acetate methods with the Ponceau S stain are more analytically accurate than agarose methods because staining intensity is nearly proportional to quantity of protein. However, accuracy is dependent on several factors, including the measured [TP], accuracy of densitometry, and the marking of protein regions. Other protein stains (e.g., Coomassie brilliant blue, amido black) have better detection limits but staining intensity is not as proportional to quantity of protein present as with the Ponceau S stain.

C. Proteins that are the major contributors to the electrophoretic pattern are shown in Fig. 7.1 and listed in Table 7.2. Migration regions are for human proteins; there is little documentation of where other mammalian proteins migrate. For some sera (e.g., bovine), only three or four globulin fractions will be detected.

D. Comments
   1. SPE has limited diagnostic value; it may be helpful to differentiate the causes of hyperproteinemia, characterize hypoproteinemias into selective or nonselective categories, screen for a monoclonal gammopathy in normoproteinemic and hyperproteinemic sera, and provide more accurate estimation of albumin concentration when globulins interfere with the BCG assay.

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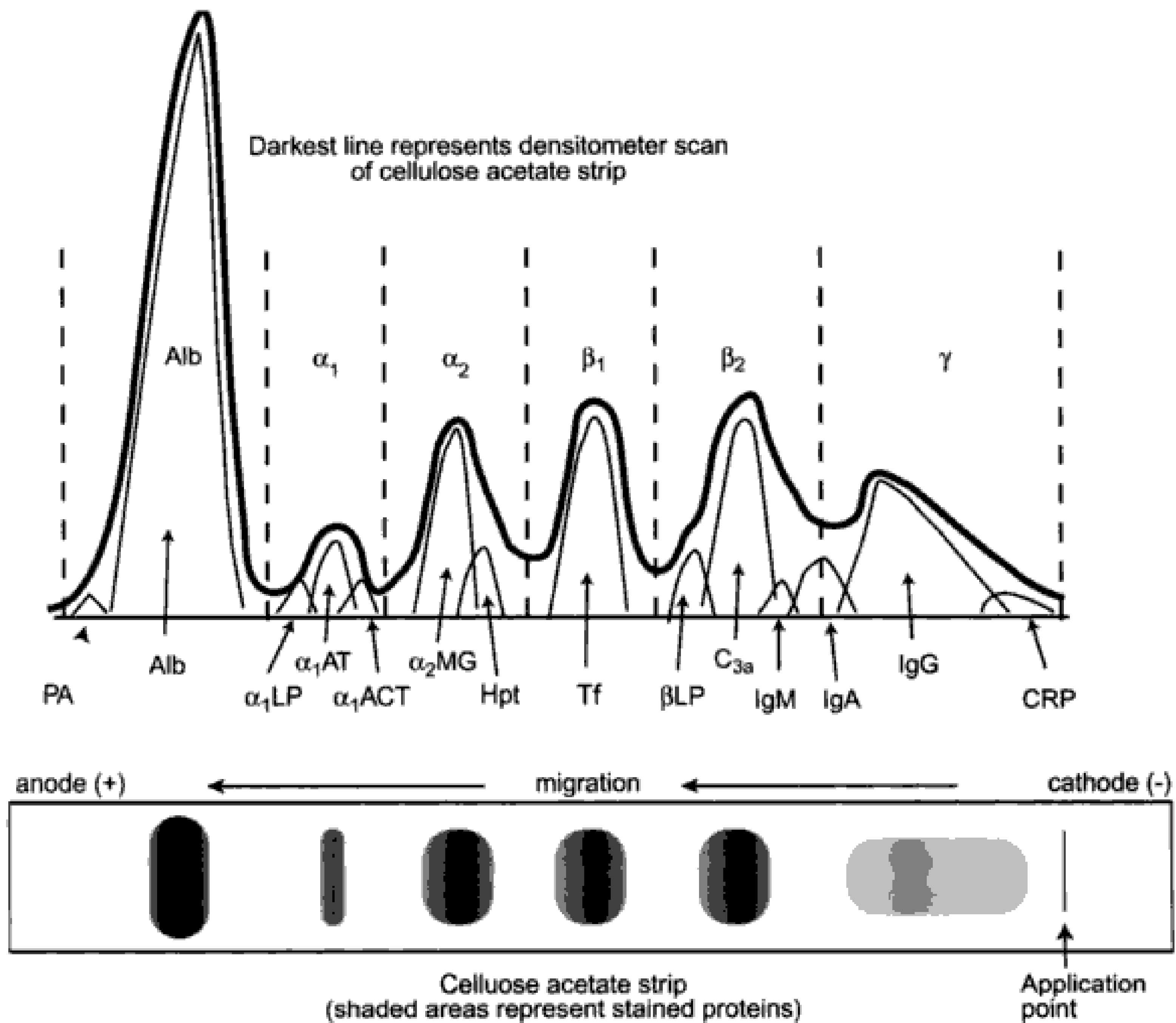

Fig. 7.1. Schematic representation of SPE results (cellulose acetate strip and densitometer tracing). (α_1ACT = a_1-antichymotrypsin, α_1AT = α_1-antitrypsin, α_1LP = α_1-lipoprotein, α_2MG = α_2-macroglobulin, βLP = β-lipoprotein, PA = prealbumin)

Proteins are separated during electrophoresis in an alkaline medium on cellulose acetate; albumin migrates the farthest toward the anode and globulin fractions separate into fractions (e.g., 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., Ponceau S); bands that contain the most protein stain the darkest. 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 are relatively lower depending on the relative staining intensities of the corresponding bands. One peak may represent the staining of one protein (e.g., albumin) or may represent the sum of multiple proteins (e.g., α_2-globulin region contains Hpt and α_2-macroglobulin).

2. Analysis of SPE is not common in clinical medicine, but understanding SPE results aids in understanding routine serum concentrations of TP, albumin, and globulins.
3. The protein bands on the cellulose acetate should be examined in addition to the densitometric findings, because the tracings do not always demonstrate the abnormalities in the protein fractions.
4. The calculated concentrations of the electrophoretic fractions are at best estimates; the true value is usually within 0.3 g/dL of the calculated value. The calculated concentrations are frequently not needed to classify or interpret dysproteinemia patterns.

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Table 7.2. Serum proteins that contribute to electrophoretic regions

Region	Proteins	M_r (in thousands)	Function and other information
Pre-alb	—	—	Not recognized in routine SPE of animal sera; includes thyroxine-binding albumin and retinol-binding protein
Alb	Albumin[a]	69	Major contributor to oncotic pressure; transports Ca^{2+}, Mg^{2+}, unconjugated bilirubin, fatty acids, thyroxine, and many other substances
α_1	α_1-lipoprotein	180–350	Transports lipids (especially cholesterol); also called HDL; relatively very low concentrations in domestic mammals when compared to people
	α_1-antitrypsin[b]	54	Inactivates proteases, including trypsin, and thus is an anti-inflammatory protein
	α_1-antichymotrypsin[b]	68	Inactivates proteases, including chymotrypsin, and thus is an anti-inflammatory protein
α_2	α_2-macroglobulin[b]	820	Inactivates proteases and thus is an anti-inflammatory protein
	Haptoglobins[b]	80–160	Bind and transport free hemoglobin
β_1	Transferrin[a]	76	Binds and transports iron; measured as TIBC in chemical assays
β_2	β lipoprotein	2400	Transports lipids (cholesterol and triglyceride); also called LDL
	Complement (C3a)[b]	180	Promotes inflammation; chemotactic substance
	IgM and IgA	IgA: 160 IgM: 900	Bind to specific antigen. Concentrations are too low in health to be seen via routine SPE.
γ	IgG	150	Binds to specific antigens. Many different isotypes and idiotypes of IgG give a broad and usually indistinct gamma region.
	C-reactive protein[b]	110	Positive acute phase protein; rarely seen in mammalian sera via routine SPE

Source: Most information is based on human plasma proteins. (Ritzmann SE, Daniels JC., eds. 1982. *Serum Protein Abnormalities: Diagnostic and Clinical Aspects.* New York: Alan R. Liss, Inc.)

Notes: Proteins in domestic mammal plasma are assumed to migrate in similar regions. There are hundreds of other plasma proteins of clinical significance but their concentrations are too low in physiologic and pathologic states to alter the electrophoretic pattern in cellulose acetate electrophoresis. If plasma is electrophoresed, fibrinogen (M_r: 341,000, a positive acute phase protein) should migrate in the cathodal end of the β_2 region.

[a] Negative acute phase protein.

[b] Positive acute phase protein.

HYPERPROTEINEMIA (INCREASED [TP] IN SERUM OR PLASMA)

The diseases and conditions that cause hyperproteinemia are given in Table 7.3.

I. Hemoconcentration

  A. Hemoconcentration is the most common cause of hyperproteinemia.

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Table 7.3. Diseases and conditions that cause hyperproteinemia

"Hemoconcentration
Increased protein synthesis
Inflammatory diseases
"Infection: bacterial, viral, fungal, protozoal
"Noninfectious disease: necrosis, neoplasia, immune-mediated disease
B-lymphocyte neoplasia
Plasma cell: multiple myeloma, plasmacytoma
Lymphocyte: lymphoma, lymphocytic leukemia

Note: All of these diseases or conditions may cause hyperglobulinemia but only hemoconcentration will cause concurrent hyperalbuminemia.

B. Pathogenesis: Hyperproteinemia results from the concentration of plasma proteins due to the loss of plasma H_2O. The plasma H_2O loss and resultant decreased ECF volume may be due to vomiting, diarrhea, impaired renal concentrating ability, sweating, insensible loss via respiration, increased vascular permeability or decreased H_2O intake combined with normal losses.

C. If proteins were the only solids in plasma, then plasma could contain about 93% H_2O and 7% proteins and the [TP] would be about 7.0 g/dL in health. If dehydration led to a 10% decrease in plasma volume, the [TP] would increase to about 7.8 g/dL (7.0/0.9 = 7.78).

D. All proteins are concentrated by loss of plasma H_2O; therefore, concentrations of albumin, globulins, and fibrinogen are proportionately increased if dehydration is the only cause of the dysproteinemia (see Plate 5.*B*).

E. Other expected laboratory findings
   1. Erythrocytosis
   2. Prerenal azotemia
   3. Hypersthenuria if renal concentrating mechanisms are functional

II. Increased protein synthesis
   A. Inflammation
      1. Inflammation is the second most common cause of hyperproteinemia.
      2. 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 interleukin-6, alter protein synthesis in or protein release from hepatocytes.[6] Cytokines primarily regulate transcription.
      3. There are three major groups of proteins in an inflammatory dysproteinemia.
         a. Positive acute phase proteins
            (1) These are proteins whose plasma or serum concentrations are increased during acute inflammation because of increased production by hepatocytes in response to inflammatory cytokines. This group includes some α_1-globulins, α_2-globulins (such as haptoglobin and α_2-macroglobulin), fibrinogen, serum amyloid A proteins, and C-reactive protein.
            (2) Production of these proteins may increase within hours and persist as long as an inflammatory process is present. Increased plasma or serum concentrations may be seen by 2 days after the onset of inflammation.
         b. Negative acute phase proteins

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(1) These are proteins whose plasma or serum concentrations are decreased during acute inflammation because of decreased production by hepatocytes. This group includes albumin and transferrin.
(2) Due to the life spans of albumin and transferrin, hypoalbuminemia and decreased TIBC (a reflection of transferrin concentration) may not be seen until inflammation has persisted for at least a week.

c. Delayed response proteins
(1) These are proteins whose plasma or serum concentrations increase 1–3 weeks after the onset of inflammation due to increased production.
(2) This group includes all immunoglobulins (IgG mostly) and complement (C3). Increased synthesis of a variety of immunoglobulins by many types (or clones) of B-lymphocytes produces a polyclonal gammopathy.

4. Expected dysproteinemia patterns
a. Acute phase response: hyperproteinemia due to acute inflammation of about 2–7 days duration
(1) Mild hyperproteinemia due to hyperglobulinemia (increased α_1- and/or α_2-globulins and hyperfibrinogenemia)
(2) Possibly mild hypoalbuminemia or low-normal serum albumin concentration
b. Delayed response: hyperproteinemia due to chronic inflammation greater than 7 days duration
(1) Hyperproteinemia (mild to marked) is due to hyperglobulinemia (increased positive acute phase and/or delayed response proteins); polyclonal gammopathy may or may not be detected by SPE.
(2) A mild to moderate hypoalbuminemia may be present.
(3) The net change in protein concentrations may produce a dysproteinemia with a lower albumin concentration and greater concentrations of some globulin fractions (see Plate 5.*C*).
c. Polyclonal gammopathy: hyperproteinemia due to chronic inflammatory and typically infectious diseases
(1) Hyperproteinemia is due to hypergammaglobulinemia (typically IgG); γ-globulin fraction may be broad-based (see Plate 5.*F*) or have a restricted migration that results in a "compact γ-globulin band" that mimics a monoclonal spike (see Plate 5.*D*).[7, 8]
(a) Polyclonal immunoglobulins consist of one or more heavy-chain classes and both light-chain classes.
(b) A polyclonal gammopathy with a restricted migration has been called an oligoclonal gammopathy. It is not a monoclonal gammopathy because its proteins do not meet the criteria for monoclonal proteins; there are increases in κ and λ light chains.
(c) The "monoclonal" gammopathies that some authors have described in animals with infectious diseases (e.g., ehrlichiosis, leishmaniasis) may have been oligoclonal gammopathies.
(2) Mild to moderate hypoalbuminemia

5. Other expected laboratory data
a. May develop anemia of inflammatory disease if inflammation persists
b. Inflammatory neutrophilia or neutropenia
c. Inflammatory lymphocytosis or lymphopenia
d. Inflammatory monocytosis

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6. Concurrent pathologic processes may complicate interpretation of the protein data. For example, there can be a concurrent increased fibrinogen production due to inflammation and increased fibrinogen consumption due to intravascular coagulation. Or, there can be concurrent inflammation and hemoconcentration or inflammation and protein-losing states.

B. B-lymphocyte neoplasia
   1. Pathogenesis: Neoplastic B-lymphocytes may produce large quantities of an immunoglobulin; typically, there is one neoplastic cell line or one clone of neoplastic lymphocytes. The single clone of lymphocytes produces an electrophoretically, structurally, and antigenically homogeneous immunoglobulin or comparably homogeneous immunoglobulin subunits. The resulting dysproteinemia is called a monoclonal gammopathy.
   2. Proteins produced by B-lymphocyte neoplasia are sometimes called M-proteins (for monoclonal proteins). "M" has also been used as an abbreviation for myeloma and macroglobulin and thus one must interpret "M-protein" in context. A monoclonal protein consists of two heavy chains of the same class (e.g., IgM) and subclass (e.g., IgG1, IgG2) and two light chains of the same type (either κ or λ, not both) (Fig. 7.2).[9] The neoplastic cells may produce intact immunoglobulins, free light chains, only heavy chains, or abnormal fragments.[10]
   3. Types of B-lymphocyte neoplasia that may cause a gammopathy
      a. Plasma cell neoplasia: multiple myeloma (most frequent cause), extramedullary plasmacytoma
      b. Lymphocyte neoplasia: lymphoma, lymphocytic leukemia
   4. Expected dysproteinemia pattern
      a. Mild to marked hyperproteinemia produced by hyperglobulinemia that contains a monoclonal gammopathy. The monoclonal protein may migrate in β- or γ-globulin fractions.
         (1) IgG typically migrates in γ-globulin fraction; IgM and IgA typically migrate at the β-γ junction or in the β-globulin fraction (see Plate 5.*G*).[11]
         (2) Atypical electrophoretic migrations may be caused by protein degradation, binding to other proteins, formation of immunoglobulin complexes, and pro-

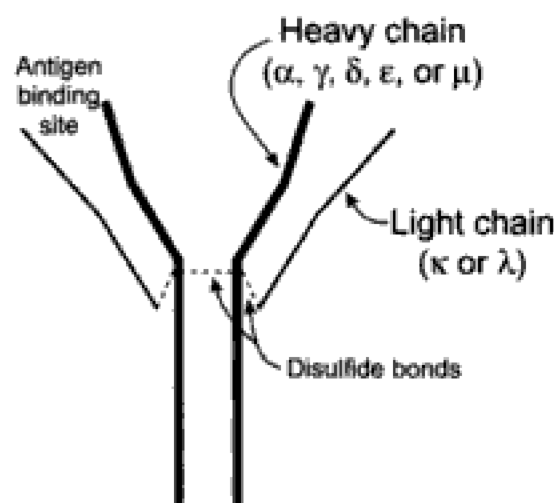

Fig. 7.2. Schematic structure of an immunoglobulin.
Immunoglobulin consists of two heavy chains of the same class (α for IgA, γ for IgG, δ for IgD, ϵ for IgE, and μ for IgM) and two light chains (either κ or λ, not both). The combination of a light chain and the slanted segment of a heavy chain is a Fab fragment that contains an antigen-binding site. The tail of the Y (vertical segments of two heavy chains) is the Fc fragment.

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duction of incomplete immunoglobulins (e.g., light chains, heavy chains, or abnormal fragments).[9]

(3) Concentrations of immunoglobulins other than the monoclonal protein frequently are decreased.

b. Mild to moderate hypoalbuminemia may be caused by decreased albumin synthesis due to inflammatory cytokines, or by a negative feedback mechanism involving an oncotic pressure receptor on hepatocytes. Increased concentration of γ-globulins (either by infusion or endogenous production) does decrease albumin synthesis, but the mechanism has not been established.[12]

5. Confirming the presence of a monoclonal gammopathy in domestic mammals is rarely accomplished.
   a. Serum electrophoresis is useful to screen for potential monoclonal spikes but frequently lacks sufficient specificity to reliably differentiate a monoclonal gammopathy from a restricted polyclonal or oligoclonal gammopathy.
      (1) Agarose electrophoresis or high-resolution electrophoretic methods will improve detection of protein bands over cellulose acetate methods but they do not provide the information needed to confirm the presence of a monoclonal gammopathy.[10]
      (2) A narrow globulin spike that is not in the γ-globulin fraction is probably a monoclonal gammopathy since such bands are usually due to either IgA or IgM and not to IgG. High concentrations of IgA or IgM are not expected in a non-neoplastic immune response.
      (3) A narrow globulin spike in the γ-globulin fraction could be a monoclonal gammopathy (due to B-lymphoid neoplasia) or an oligoclonal (or restricted polyclonal) gammopathy (due to an immune response).
   b. Immunoelectrophoresis
      (1) If a monoclonal protein is suspected, then immunoelectrophoresis with species-specific anti-IgG (including subclasses), anti-IgM, anti-IgA, anti–κ chain, or anti–λ chain is needed to differentiate monoclonal and polyclonal gammopathies.[9] Unfortunately, such antisera are rarely available for domestic mammals.
      (2) If it is a monoclonal gammopathy, then the increased concentration of one heavy-chain class (or subclass) will be associated with either κ or λ chains because a lymphocyte clone produces either κ or λ chains, not both.[11]
   c. Immunofixation is reported to have greater sensitivity than immunoelectrophoresis but requires monospecifc antisera to IgG, IgM, IgA, and free and bound κ or λ chains.[10]
   d. SRID
      (1) SRID 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.
      (2) SRID assays for total [IgG] cannot be used to differentiate an IgG polyclonal gammopathy from an IgG monoclonal gammopathy.

6. Associated laboratory or clinical problems
   a. Bence Jones proteinuria (BJ proteins = light chains of immunoglobulins)
      (1) Bence Jones proteinuria may occur because there is an increased formation of κ or λ light chains by lymphoid cells. Light chains (M_r: ≈ 23,000 monomer or 46,000 dimer) pass through the glomerular filtration barrier and small quantities are resorbed by proximal tubules. If renal resorptive capacity is

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exceeded or if renal disease develops, light chains are excreted in urine. If urine proteins are concentrated prior to electrophoresis, light chains can be found in the β_2-globulin fraction (see Plate 5.*J*.2).

(2) The Bence Jones proteins do not react strongly with the protein reagent pad of routine urinalysis procedures but do with the sulfosalicylic acid procedures.[11] Accordingly, the reagent pad test may fail to detect a significant proteinuria (see Chap. 8).

(3) Confirmation of a Bence Jones proteinuria is attempted by two methods.

(a) Bence Jones test:[13] Bence Jones proteins have unique thermal properties; they precipitate between 40°C and 60°C, return to a soluble state at 100°C, then precipitate again when cooled. Bence Jones protein concentration needs to be > 145 mg/dL to get a positive result.[11] Other proteins may precipitate and make interpretation difficult; these proteins may be removed by filtering the hot urine and then the filtrate can be used for the Bence Jones test. The thermal properties of Bence Jones proteins are due to the variable portions of the light chains.[11]

(b) Immunoelectrophoresis for κ or λ light chains is the preferred method, but is not readily available for individual species.

b. Hyperviscosity syndrome

(1) High immunoglobulin concentrations may cause the plasma to become viscous. 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.

(2) The high plasma protein concentration may also cause abnormal platelet function.

(3) The hyperviscosity syndrome is seen with high concentrations of IgA, IgG, or IgM.

7. Comments

a. An increased concentration of an immunoglobulin is called a gammopathy regardless of where the immunoglobulin migrates with SPE (it could be in α-, β-, or γ-globulin fractions).

b. Some authors have reported that infections (e.g., ehrlichiosis) can cause a monoclonal gammopathy because a narrow-based spike was seen in densitometer scans of routine SPE. The observed spike may have represented a compact γ-globulin band (called an oligoclonal or a restricted polyclonal gammopathy)[7] in which many IgG types migrated in the same restricted space. It is very unlikely that an infection with complex organisms such as *Ehrlichia* would result in the stimulation of only one clone of lymphocytes to produce a monoclonal gammopathy with only κ or λ light chains.

HYPOPROTEINEMIA (DECREASED [TP] IN SERUM OR PLASMA)

The diseases and conditions that cause hypoproteinemia are given in Table 7.4.

I. Increased protein loss from 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

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Table 7.4. Diseases and conditions that cause hypoproteinemia

Increased protein loss from vascular space
- [a]Blood loss
- [a]Protein-losing nephropathy: glomerulonephritis, amyloidosis
- [a]Protein-losing enteropathy: small intestinal mucosal disease, lymphangiectasia, intestinal blood loss
- Protein-losing dermatopathy: burns, generalized exudative skin disease

Decreased protein synthesis and/or increased protein catabolism
- [a]Hepatic insufficiency
- [a]Malabsorption or maldigestion: intestinal mucosal disease, exocrine pancreatic insufficiency
- [a]Cachectic states: chronic diseases, neoplasia, malnutrition, starvation
- Lymphoid hypoplasia or aplasia

Failure of passive transfer (FPT)

Hemodilution
- Excess administration of intravenous fluid
- Edematous disorders: congestive heart failure, cirrhosis, nephrotic syndrome
- Excess ADH secretion: SIADH

Note: Total protein concentrations in healthy pups, kittens, calves, and foals may be 1–2 g/dL less than those found in mature animals.

(see Mechanism of Acute External Blood Loss Anemia in Chap. 4). Hypoproteinemia will persist as long as the rate of loss exceeds the rate of protein production.

2. Major laboratory findings
   a. Panhypoproteinemia: decreased [TP] with decreased concentrations of albumin and globulins; a nonselective hypoproteinemia
   b. Anemia: The type of anemia depends on chronicity of blood loss (see Chap. 4).

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. When proteins enter the filtrate at a rate greater than proximal tubules can resorb, then proteinuria occurs. When the rate of protein loss exceeds protein production, hypoproteinemia occurs. The largest proteins (e.g., α_2-macroglobulin and β-lipoprotein) typically are not lost through glomeruli and thus a 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 a PLN.
2. Diseases: glomerulonephritides, amyloidosis
3. Major laboratory findings
   a. Mild to marked hypoproteinemia with hypoalbuminemia and normoglobulinemia (occasionally hypoglobulinemia)
   b. SPE results: selective hypoproteinemia pattern with α_2-globulins WRI or only mildly decreased, but other protein fractions definitely decreased (see Plate 5.*I*.1)
   c. Moderate to marked proteinuria dominated by albuminuria (see Plate 5.*I*.2)
   d. Perhaps evidence of renal insufficiency (azotemia, isosthenuria) if the disease has destroyed enough nephrons
   e. Hypercholesterolemia and abdominal transudate if nephrotic syndrome

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C. PLE
1. Pathogenesis
a. Intestinal secretions are relatively protein-rich, typically are digested and absorbed in the small intestine, and then are transported to the portal system and lymphatic vessels. When generalized small intestinal mucosal diseases or lymphatic diseases prohibit the absorption or transport of the proteins, they are lost in feces. When the rate of protein loss exceeds the ability of the liver and lymphocytes to produce proteins, hypoproteinemia occurs.
b. In some disorders, inflammatory exudation and decreased protein intake contribute to the hypoproteinemia.
c. Intestinal blood loss due to parasitism is one form of PLE.
2. Diseases
a. Generalized small intestinal mucosal diseases: lymphoma, histoplasmosis, lymphocytic/plasmacytic/eosinophilic enteritis
b. Horses with acute enteritis
c. Lymphatic disease: lymphangiectasia, lymphosarcoma
d. Intestinal blood loss: hookworms, whipworms, neoplasia
3. Major laboratory findings
a. Mild to marked hypoproteinemia with hypoalbuminemia and hypoglobulinemia (or normoglobulinemia)
b. SPE results: usually a nonselective pattern but may be selective
c. Other findings may indicate or suggest the inciting pathologic state (e.g., *Histoplasma* organisms or neoplastic lymphocytes in biopsy samples; melena or other evidence of blood loss).

D. Protein-losing dermatopathy
1. Pathogenesis
a. Thermal or chemical burns allow plasma proteins to exude from cutaneous lesions at a rate greater than the rate of protein production. If not seen soon after the injury, the dysproteinemia will reflect a mixture of cutaneous protein loss and an acute phase inflammatory response.[14]
b. Generalized exudative skin disease can contribute to the hypoproteinemic state, but the dysproteinemia may reflect a mixture of protein loss and an inflammatory dysproteinemia.
2. Major laboratory findings
a. Early: nonselective hypoproteinemia
b. Later: nonselective hypoproteinemia masked by either an acute or chronic inflammatory dysproteinemia

II. Decreased protein synthesis and/or increased protein catabolism
A. Hepatic insufficiency (or hepatic failure)
1. Pathogenesis: Marked reduction in functional hepatic mass (< 20% remaining) results in decreased synthesis of nearly all plasma proteins except immunoglobulins. Normal protein catabolism combined with decreased protein synthesis produces the hypoproteinemia.
2. Disorders
a. Cirrhosis
b. Hepatic necrosis or inflammation (not acute)
c. Hepatic atrophy secondary to portosystemic shunts

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d. Neoplasms that cause extensive damage to liver

3. Major laboratory findings
   a. Hypoproteinemia, hypoalbuminemia, and normoglobulinemia or hypoglobulinemia
   b. SPE results: typically a nonselective pattern; possibly relative excess in β_2- or γ-globulins because of one of two theories
      (1) There may be a compensatory increased synthesis of immunoglobulins to attempt to maintain a colloidal or oncotic pressure in the vascular system.
      (2) A liver removes antigenic material and IgA from the portal blood. With hepatic insufficiency, the antigenic material gains entrance to peripheral blood and induces a systemic immune response (increased IgM, IgA, or IgG). Because IgM and IgA migrate in the β_2-globulin fraction and IgG in the γ-globulin fraction, there may not be a clear distinction between β_2- and γ-globulin fractions (called a beta-gamma bridge).
4. Other chemical findings of hepatic disease or dysfunction: increased hepatic enzyme activities, decreased urea concentration, increased bile acid or ammonium concentrations

B. Malabsorption or maldigestion
1. Pathogenesis: A malabsorptive or maldigestive state results in a deficient intake of basic body fuels (carbohydrates, proteins, or fats) to replace fuels used by metabolic pathways for daily energy. Once depleted, protein catabolism and use of amino acids for gluconeogenesis leads to a deficiency in energy and amino acids for hepatocellular and lymphocytic protein synthesis. When catabolism exceeds production, hypoproteinemia occurs.
2. Disorders
   a. Malabsorption: small intestinal diseases where generalized mucosal involvement causes malabsorption of digested proteins, carbohydrates, and lipids
   b. Maldigestion: exocrine pancreatic deficiency (due to chronic pancreatitis or pancreatic atrophy) creates deficiencies in proteases, lipase, and amylase and thus maldigestion of proteins, lipids (fat), or carbohydrates (starches)
3. Major laboratory findings
   a. Hypoproteinemia, hypoalbuminemia, and normoglobulinemia or hypoglobulinemia
   b. SPE results: typically a nonselective pattern (see Plate 5.*H*)
   c. Other findings dependent on primary pathologic state (e.g., decreased trypsin-like immunoreactivity with exocrine pancreatic insufficiency, poor xylose absorption with malabsorptive states [see Chap. 15])

C. Cachectic states
1. Pathogenesis: When the rate of protein catabolism exceeds protein production, the negative protein status results in a hypoproteinemia. Before a clinical hypoproteinemia develops, the animal attempts to maintain glucose and most serum proteins (especially albumin) at the expense of other tissues. Therefore, this hypoproteinemia is expected when there has been loss of body weight due to decreased fat and muscle mass.
2. Disorders
   a. Chronic diseases such as chronic infections and malignant neoplasia
   b. Marked malnutrition or starvation
3. Major laboratory findings

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a. Hypoproteinemia, hypoalbuminemia, and normoglobulinemia or hypoglobulinemia
b. SPE results: typically a nonselective pattern
c. Other findings dependent on primary pathologic state

D. Lymphoid hypoplasia or aplasia
1. B-lymphocytes produce immunoglobulins and not other major plasma proteins. A mild hypoproteinemia can be created by lymphoid hypoplasia if concentrations of other proteins are WRI.
2. Expected dysproteinemia
a. [TP] is WRI to slightly decreased. Albumin concentration is WRI, and globulin concentration is WRI or slightly decreased.
b. SPE results: decrease in γ-globulin concentration
3. Disorders
a. Combined immunodeficiency in horses (Arabian, Appaloosa) and dogs (Basset hound, Cardigan Welsh corgi, Jack Russell terrier): need quantitative immunoglobulin techniques to confirm decreases or deficiencies of immunoglobulins
b. Chemotherapy or infections causing lymphoid hypoplasia

III. FPT
A. Neonates who fail to ingest or absorb colostral antibodies will have lower serum or plasma [TP] because of lower [IgG]. However, inflammation or dehydration can increase the [TP] in such animals and thus mask the FPT.
B. This relationship between [IgG] and [TP] has been explored and resulted in the following findings in sera from 1- to 8-day-old calves when compared to an [IgG] decision value of 1000 mg/dL.[15]
1. With [sTP_{ref}] of 5.0 g/dL as a decision value, 83% were correctly classified regarding passive transfer status.
2. With [sTP_{ref}] of 5.5 g/dL as a decision value, 82% were correctly classified regarding passive transfer status.

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, nephrotic syndrome)
3. Excess ADH secretion (SIADH)

HYPERALBUMINEMIA

The diseases and conditions that cause hyperalbuminemia are given in Table 7.5.

Table 7.5. Diseases and conditions that cause hyperalbuminemia

[a]Hemoconcentration
Increased albumin synthesis induced by glucocorticoid drugs or hormones

Note: If albumin concentration is determined by the BCG method, the BCG dye may bind to proteins other than albumin and thus give a pseudohyperalbuminemia.

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I. Hemoconcentration (dehydration): Decreased ECF volume
   A. Decreased plasma H_2O leads to greater concentrations of those substances (including albumin) 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.

II. Induced synthesis by glucocorticoid therapy
   A. Glucocorticoid therapy can cause hyperalbuminemia in dogs[16-18] and in cats (unpublished data). In dogs, albumin concentration increased about 2 g/dL after 4–5 days of treatment with methylprednisolone (4 mg/kg IM per day).[16] Also in dogs after 4 weeks of treatment with prednisone (0.55 mg/kg, q12hr), albumin concentration increased about 0.8 g/dL along with a concurrent increase in haptoglobin concentration.[17]
   B. Increased albumin concentration may be the result of increased production[19] or possibly increased life span.

III. Falsely increased concentration determined by BCG dye method
   A. BCG dye preferentially binds to albumin. However, it also binds to some other proteins and thus a measured albumin concentration represents the dye binding to albumin and some globulins. One globulin that binds BCG dye is orosomucoid (α_1-acid glycoprotein).
   B. This pseudohyperalbuminemia is seen most frequently in equine and swine sera, but it also occurs in other sera. The anomalous albumin concentration may not be recognized in hypoalbuminemic samples; i.e., the hypoalbuminemia is more severe than indicated by the measured concentration.

HYPOALBUMINEMIA

The diseases and conditions that cause hypoalbuminemia are given in Table 7.6.

Table 7.6. Diseases and conditions that cause hypoalbuminemia

Decreased albumin synthesis
[a]Inflammation
[a]Hepatic insufficiency
[a]Malabsorption and maldigestion
[a]Cachectic states
Hypergammaglobulinemia
Increased albumin loss
[a]Blood loss
[a]Protein-losing nephropathy: glomerulonephritis, amyloidosis
[a]Protein-losing enteropathy: small intestinal mucosal disease, lymphangiectasia, intestinal blood loss
Protein-losing dermatopathy: burns, generalized exudative skin disease
Hemodilution
Excess administration of intravenous fluid
Edematous disorders: congestive heart failure, cirrhosis, nephrotic syndrome
Excess ADH secretion: SIADH

Note: Albumin concentrations in healthy pups, kittens, calves, and foals may be 0.5–1.0 g/dL less than those found in mature animals.

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I. Hypoalbuminemia commonly occurs with hyperproteinemia and hypoproteinemia, but it also can be seen when there is normoproteinemia.

II. Pathogeneses of the hypoalbuminemic states are described in the appropriate dysproteinemia sections (see sections Hyperproteinemia, II; and Hypoproteinemia above).

HYPERGLOBULINEMIA (See Hyperproteinemia section above.)

HYPOGLOBULINEMIA (See Hypoproteinemia section above.)

FIBRINOGEN

I. Hyperfibrinogenemia (plasma) (Table 7.7)
   A. Two major causes
      1. Hemoconcentration: decreased plasma H_2O
      2. Inflammation: increased fibrinogen production by liver
   B. [TP] is usually also increased with these disorders but may be WRI. Usually, fibrinogen concentration increases relatively more than [TP] in inflammation. With dehydration, the increases in [TP] and fibrinogen concentration should be relatively the same; e.g., they both increase by 5%. These concepts led to the development of the PP:F ratio (Eq. 7.1.a).[20] The ratio was simplified a few years later by not subtracting fibrinogen concentration from the $[TP_{ref}]$ (Eq. 7.1.b).[21]

$$\text{PP:F ratio} = \frac{[\text{plasma TP}] - [\text{fibrinogen}]}{[\text{fibrinogen}]} \qquad \textbf{(7.1.a.)}$$

$$(\text{TP:Fib})_p \text{ ratio} = \frac{[\text{plasma TP}]}{[\text{fibrinogen}]} \qquad \textbf{(7.1.b.)}$$

$$\text{Example: } [\text{pTP}] = 8.8 \text{ g/dL} \;\&\; [\text{fibrinogen}] = 0.8 \text{ g/dL}$$

$$\text{PP:F ratio} = \frac{8.8 \text{ g/dL} - 0.8 \text{ g/dL}}{0.8 \text{ g/dL}} = \frac{8.0 \text{ g/dL}}{0.8 \text{ g/dL}} = 10$$

$$(\text{TP:Fib})_p \text{ ratio} = \frac{8.8 \text{ g/dL}}{0.8 \text{ g/dL}} = 11$$

   C. PP:F ratio or $(\text{TP:Fib})_p$ ratio
      1. Purpose: attempt to differentiate hyperfibrinogenemias of inflammation and dehydration
      2. The original interpretive guides and normal values were established with the PP:F ratio.[20,22] The PP:F ratios in healthy mammals varied between species and by age group, mostly because of different total protein concentrations.[23]
         a. Cattle: If the ratio is > 15, hyperfibrinogenemia is probably due to dehydration; if the ratio is < 10, hyperfibrinogenemia is probably due to inflammation.

Table 7.7. Diseases and conditions that cause hyperfibrinogenemia

Increased fibrinogen production
*Inflammation
*Hemoconcentration

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b. Horses: If the ratio is > 20, hyperfibrinogenemia is probably due to dehydration; if the ratio is < 15, hyperfibrinogenemia is probably due to inflammation.
c. If the simpler calculation, $(TP:Fib)_p$ 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).

3. Guidelines are based on the assumptions that healthy animals TP and fibrinogen concentrations are WRI and then one of two things happens: the animal becomes dehydrated or develops an inflammatory disease.
   a. Dehydration will cause increased concentrations of all proteins to the same degree and thus the PP:F and $(TP:Fib)_p$ ratios do not change.
   b. Inflammation will cause increased concentrations of fibrinogen and some proteins but also decreased concentrations of other proteins. Thus, the PP:F and $(TP:Fib)_p$ ratios will decrease because the denominator will increase relatively more than the numerator.
4. Additional factors to be considered
   a. Concurrent dehydration and inflammation will make interpretation of the ratios more difficult. Increased fibrinogen consumption in the pathologic state will also cloud the issue.
   b. Dehydration by itself will cause only minor increases in fibrinogen concentration.
   c. The above ratio guidelines are not appropriate for calves and foals because the ratio reference intervals are for mature animal values. They also are not appropriate for cattle and horses that have a concurrent pathologic state that causes hypoproteinemia.
   d. The accuracy of refractometric values is at best ± 0.1 g/dL. Thus the accuracy of the calculated ratio is probably better with higher fibrinogen concentrations. Even then, however, they should be considered estimates.

D. Why are fibrinogen concentrations and PP:F ratio or $(TP:Fib)_p$ ratio used in cattle and horses?
   1. Leukogram changes in inflammatory conditions frequently are mild and may not indicate the presence of an inflammatory disease. Hyperfibrinogenemia may be present whether or not the leukogram reflects an inflammatory state.
   2. The PP:F ratio or the $(TP:Fib)_p$ ratio is used to attempt to determine the contribution of dehydration to the hyperfibrinogenemic state.

II. Hypofibrinogenemia (plasma) (Table 7.8)
   A. Heat precipitation techniques lack sufficient analytical sensitivity and precision to be used for detection or confirmation of hypofibrinogenemia. Quantitative assays are needed for documentation of hypofibrinogenemia.
   B. Causes of hypofibrinogenemia (more information in Chap. 5)

Table 7.8. Diseases and conditions that cause hypofibrinogenemia

Increased fibrinogen consumption
[a]Intravascular coagulation (localized or disseminated)
Increased fibrinogenolysis
Decreased synthesis of fibrinogen
Hepatic insufficiency
Afibrinogenemia (congenital or inherited)
Dysfibrinogenemia

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1. Increased consumption of fibrinogen
   a. Intravascular coagulation: local or disseminated thrombi formation or vasculitis
   b. Increased fibrinogenolysis: DIC
2. Decreased synthesis of fibrinogen
   a. Hepatic insufficiency: must be marked reduction in hepatic function before clinically significant hypofibrinogenemia occurs
   b. Inherited or congenital disorders: afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia
      (1) The common assay for detecting hypofibrinogenemia is the thrombin time, or its Clauss modification (see Chap. 5).
      (2) Afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia have been reported in dogs, but the antigenic assays needed to differentiate the true and functional deficiencies have not always been done. Fibrinogen deficiencies have been found in the Bichon Frise,[24] Bernese mountain dog (Berner sennenhund),[25] and the Lhasa apso, vizla, and collie.[26] Some of the dogs had prolonged hemorrhage after the hemostasis system was challenged.

IMMUNOGLOBULIN G (IgG)

I. The most common reason for measuring [IgG] is to determine if there has been successful passive transfer of IgG from a mother (mare or cow) to her foal or calf via colostrum. Accordingly, passive transfer will be the focus of this section. Immunoglobulin concentrations, i.e., IgG, IgM, IgA, IgG(T), may also be measured when evaluating congenital and acquired immunodeficiency states or when assessing potential monoclonal gammopathies.

II. Physiologic processes
   A. Placentation in horses and cattle prevents *in utero* transfer of immunoglobulins to the fetus, so neonatal horses and ruminants must ingest colostrum soon after birth (before "gut closure") to obtain maternal immunoglobulins, especially IgG. After ingestion and absorption, the half-life of maternal IgG is about 20–30 days in foals[27] and 20 days in calves.[28] Inadequate immunoglobulin transfer results in increased risk of infectious diseases and decreased rate of gain.
   B. Neonatal foals prior to colostrum have essentially no IgG in their plasma; calves have very low concentrations. Once exposed to antigens, the neonatal foal is stimulated to produce IgG and protective immunity in about 10–14 days.[27] Synthesis of IgG by calves can be detected by 8–16 days.[29]
   C. Fetal foals and calves have limited ability to produce IgM and thus neonatal foals and calves should have low concentrations of IgM.

III. Causes of FPT
   A. Lack of colostrum ingestion (neonate is too weak or because of other factors)
   B. Inadequate IgG in colostrum (decreased concentration or inadequate volume of colostrum)
   C. Failure to absorb ingested colostrum (ingested after "gut closure")

IV. Establishing presence or absence of passive transfer
   A. Foal guidelines. Blood samples are collected between 18 and 48 hr after birth.
      1. For several years, [IgG] > 400 mg/dL was considered evidence of passive transfer.

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However, [IgG] > 800 mg/dL may be a better criterion and is currently recommended.[27, 31]

2. [IgG] < 200 mg/dL is considered evidence of FPT.
3. [IgG] between 200 mg/dL and 800 mg/dL is considered as evidence of partial FPT. Such concentrations may not provide adequate humoral protection, especially for foals with higher risk factors.

B. Calf guidelines are not firmly established; two published guidelines follow. For each, blood samples are collected between 1 and 8 days after birth.
   1. One recommendation[15,32]
      a. [IgG] > 1000 mg/dL is considered evidence of passive transfer.
      b. [IgG] < 500 mg/dL is considered evidence of FPT.
   2. Another recommendation[33,34]
      a. [IgG] > 1600 mg/dL is considered evidence of passive transfer.
      b. [IgG] = 800–1600 mg/dL is considered partial passive transfer.
      c. [IgG] < 800 mg/dL is considered FPT.

C. Results for blood samples collected from older foals or calves, especially if ill, will be more difficult to interpret since the half-life of maternal IgG and a neonate's response to antigens after birth must be considered.

V. Methods of measuring or estimating [IgG] in foals and calves

A. Because of the lack of standardization of assay methods and reference standard sera, there may be marked variations in IgG concentrations measured by different IgG assays. Thus, the interpretive guidelines will vary.

B. SRID test for foal or calf serum
   1. Principle: IgG diffuses in a gel containing anti-equine or anti-bovine IgG for 18–24 hr. The diameter of a precipitant ring is proportional to the [IgG] in serum.
   2. Results: SRID is considered to be a quantitative assay for measuring [IgG].
   3. Comments
      a. Usually SRID is considered too time consuming (24-hr diffusion time) and expensive for routine clinical use.
      b. SRID has been considered the gold standard method in veterinary laboratories; however, some commercial SRID assays overestimate [IgG], especially at [IgG] > 2000 mg/dL.[35] Also, some published [IgG] values for neonates appear to be unexpectedly high if compared to expected [TP].

C. Glutaraldehyde coagulation test for foal or calf serum
   1. Principle: Glutaraldehyde (10%) promotes the formation of molecular cross-linkages to coagulate basic proteins such as immunoglobulins and fibrinogen. Because fibrinogen is absent in serum and very little IgM is present in neonatal foal serum, the amount of coagulated protein is primarily dependent on [IgG].
   2. Results
      a. For estimation of equine [IgG][36,37]
         (1) Reported semiquantitative values (gelling times may vary with newer assays)
            (a) [IgG] ≥ 800 mg/dL if serum forms gel ≤ 10 min.
            (b) [IgG] > 400 mg/dL if serum forms gel ≤ 60 min.
            (c) [IgG] ≤ 400 mg/dL if serum did not gel by 60 min.
         (2) Excellent agreement with SRID assay when [IgG] < 400 mg/dL and very good agreement with SRID assay when [IgG] > 800 mg/dL.[36]
      b. For estimation of bovine [IgG]

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(1) Semiquantitative values[34]
  (a) [IgG] > 600 mg/dL if serum forms firm opaque clot by 60 min.
  (b) [IgG] ≈ 400–600 mg/dL if serum forms semisolid gel by 60 min.
  (c) [IgG] < 400 mg/dL if serum did not gel by 60 min.
(2) Comments
  (a) To determine [IgG] at the decision limits for FPT (either > 1000 or > 1600 mg/dL), serum would need to be diluted prior to analysis.
  (b) Results of the glutaraldehyde coagulation test (Gamma-check B) were unreliable when whole blood was used.[38]

3. Very inexpensive and simple test because it just requires glutaraldehyde, reaction tubes, and pipettes; does require that serum be harvested from blood.

D. Latex agglutination (Foalcheck) for foal serum
  1. Principle: Latex beads coated with anti-equine IgG will agglutinate in the presence of equine IgG.
  2. Results: It is a semiquantitative assay that generally agrees with SRID assay results but is not as good as the glutaraldehyde coagulation test.[36]
  3. Comments: The test is moderately expensive but only takes about 10 min to complete after serum is collected.

E. $ZnSO_4$ turbidity test for foal or calf serum
  1. Principle: Sulfates selectively precipitate cationic proteins such as immunoglobulins; other proteins are neutral or negatively charged. At a constant [SO_4], the quantity of precipitate (greater turbidity) corresponds to higher immunoglobulin concentrations. Since there is very little IgA or IgM in foal or calf sera, the amount of turbidity reflects the [IgG] in sample.
  2. Results can be assessed visually or turbidimetrically.
    a. Turbidimetric assessment requires a spectrophotometer and standard solutions to establish a standard curve.
    b. In foals, visual turbidity occurs when [IgG] is near 400–500 mg/dL, which does not match with the current decision limits for FPT.[39]
    c. In calves:
      (1) With a $ZnSO_4$ solution at 208 mg/L, sufficient turbidity to obscure newsprint occurs when [IgG] > 1600 mg/dL.[34]
      (2) Results with a $ZnSO_4$ solution (208 mg/L) were compared with RID assay results.[15]
        (a) Inadequate turbidity was found in samples with [IgG] values that ranged from 0 to 2825 mg/dL (mean of 955 mg/dL). Adequate turbidity (newsprint not legible) was found in samples with [IgG] values that ranged from 1085 to 4305 mg/dL (mean of 2219 mg/dL).
        (b) The marked variations in the results suggest that the assays were too inaccurate to allow confident decisions regarding passive transfer.
      (3) With higher concentrations of $ZnSO_4$ (250–400 mg/L), lower [IgG] produces the same degree of turbidity as higher [IgG] using 200 mg/dL $ZnSO_4$.[41]
  3. Comments
    a. $ZnSO_4$ reagents may be made from scratch or purchased in kits. Stock solutions need to be sealed to prevent CO_2 absorption.
    b. For foals, turbidity tests have essentially been replaced by more accurate and convenient assays. The $ZnSO_4$ turbidity test tends to underestimate [IgG] when > 400 mg/dL.[40]

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c. For calves, the $ZnSO_4$ turbidity test provides a good estimate of [IgG] but was not considered to be as useful as the Na_2SO_3 precipitant test.[34]
d. Presence of Hgb from hemolysis will falsely increase the [IgG] if turbidity is assessed by spectrophotometry (@ 660 nm); Hgb-induced increments are about 200 mg/dL at 1% hemolysis and 1300 mg/dL at 5% hemolysis.

F. Na_2SO_3 precipitant test for calf serum
1. Principle: Sulfites selectively precipitate cationic proteins such as immunoglobulins; other proteins are neutral or negatively charged. Higher concentrations of sulfites have greater ability to precipitate lower [IgG] concentrations. Since there is very little IgA or IgM in calf sera, the amount of turbidity reflects the quantity of IgG in the sample.
2. Guidelines for interpretation of results with 14%, 16%, and 18% Na_2SO_3 solutions.[34] (Note: Estimated concentrations do not match with some recommended decision limits and thus would be difficult to interpret; see earlier section.)[33]
a. If [IgG] > 1500 mg/dL, precipitates are seen in 14%, 16%, and 18% solutions.
b. If [IgG] ≈ 500–1500 mg/dL, precipitates are seen in 16% and 18% solutions.
c. If [IgG] < 500 mg/dL, precipitate is seen in the 18% solution.
3. In another study, results were compared with [IgG] from a RID assay.[15] (Note: The marked variation in these results highlights the potentially poor accuracy or lack of precision of such assays.)
a. [IgG] ranged from 0 to 2400 mg/dL with no precipitate.
b. [IgG] ranged from 645 to 2450 mg/dL with precipitate in the 18% solution.
c. [IgG] ranged from 1025 to 4305 mg/dL with precipitate in 16% and 18% solutions.
d. [IgG] ranged from 2380 to 3625 mg/dL with precipitate in 14%, 16%, and 18% solutions.
4. Comments
a. Na_2SO_3 precipitant test is a relatively inexpensive and quick test; the major advantage over $ZnSO_4$ is the ability to estimate [IgG] in a broad range.
b. Compared to the RID assay results, the precipitant test is at best a semiquantitative assay.
c. The assay does not work well for foal serum.

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Chapter 8

URINARY SYSTEM

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Table 8.1. Abbreviations and symbols in Chapter 8

»	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
1,25-DHCC	1,25-dihydroxycholecalciferol
AcCoA	acetyl CoA
ADH	antidiuretic hormone (arginine vasopressin)
AMP	adenosine monophosphate
AMS	amylase
ANP	atrial natriuretic peptide
ATPase	adenosine triphosphatase
Bc	conjugated bilirubin
Bu	unconjugated bilirubin
Ca^{2+}	calcium
CBB	Coomassie brilliant blue
CRH	corticotrophin releasing hormone
Crt	creatinine
$Crt_u:Crt_p$	creatinine concentration of urine to creatinine concentration of plasma
DDAVP	1-deamino-8-D-arginine vasopressin
ECF	extracellular fluid
F.E.	fractional excretion
fCa^{2+}	free ionized calcium (not bound or complexed)
fMg^{2+}	free ionized magnesium (not bound or complexed)
GFR	glomerular filtration rate
GGT	γ-glutamyltransferase
hpf	high power field (400× magnification)
lpf	low power field (10× magnification)
LPS	lipase
LVP	lysine vasopressin
M_r	relative molecular weight
NADH	reduced nicotinamide adenine dinucleotide
NH_3	ammonia
NH_4^+	ammonium
$Osm_u:Osm_p$	osmolality of urine to osmolality of plasma
$Osm:USG_{ref}$	osmolality to urine specific gravity by refractometry (see text)
PD	polydipsia
Pi	inorganic phosphorus
PO_4	inorganic phosphate (all forms)
$(Prot:Crt)_u$	urinary protein concentration to creatinine concentration
PTH	parathyroid hormone
PU	polyuria
RBC	erythrocyte
RPF	renal plasma flow
sd	standard deviation
SEM	standard error of the mean
SI	Système International d'Unités
SSA	sulfosalicylic acid
TCA	trichloroacetic acid

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tCa^{2+}	total calcium
UA	urinalysis (urine analysis)
UN	urea nitrogen
$(UN:Crt)_s$	urea nitrogen concentration to creatinine concentration in serum
$UN_u:UN_p$	urea nitrogen concentration of urine/urea nitrogen concentration of plasma
USG_{ref}	refractometric urine specific gravity
vit. D	vitamin D (all forms)
WBC	leukocyte
WRI	within reference interval

PHYSIOLOGIC PROCESSES

I. Three major processes control renal excretion of H_2O and solutes: glomerular filtration (passive), tubular resorption (passive or active), and tubular secretion (active).

II. Glomerular filtration
- A. A major route for solute and H_2O excretion from an animal is through renal glomeruli. The glomerular filtration barrier is composed of capillary endothelium, basement membrane, and glomerular epithelial cells (podocytes) with foot processes (Fig. 8.1). The filtering function of glomeruli is typically assessed by evaluating the renal excretion of substances that pass freely through the glomerular filtration barrier.
  1. The ability of a substance to pass through the healthy glomerular filtration barrier (from plasma to filtrate) is dependent on two major factors.
     a. Molecular size: If < 2.5 nm radius, nearly 100% passes through; if > 3.4 nm radius, almost none passes 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.

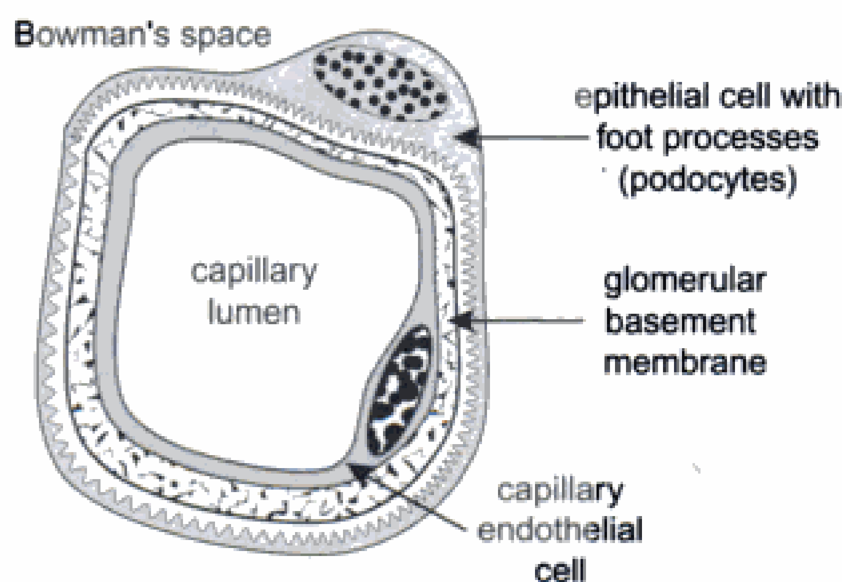

Fig. 8.1. Glomerular filtration barrier.
The glomerular filtration barrier consists of capillary endothelial cell, glomerular basement membrane, and epithelial cells (podocytes). H_2O and most solutes pass through fenestrations in the endothelial cells, through a semipermeable basement membrane, through the slit pores between foot processes of the podocytes, into Bowman's space, and then into the proximal renal tubule.

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2. In many species, albumin (M_r = 69,000; 3.5 nm radius) is near the cutoff size of the filtration barrier; its negative charge also impedes transit. Albumin is not expected in the urine of cats, horses, or cattle; a small amount may be found in the urine of healthy dogs.

B. GFR
1. GFR is the rate fluid moves from plasma to glomerular filtrate; it is measured by determining the rate a substance is cleared from plasma.
2. GFR is primarily dependent on the rate of RPF, and it varies proportionately with RPF. RPF is dependent on blood volume, cardiac output, number of functional glomeruli, and constriction or dilation of afferent and efferent glomerular arterioles. Other factors that affect GFR include intracapsular hydrostatic pressure in Bowman's space (if increased, decreased GFR) and plasma oncotic pressure (if decreased, increased GFR).
3. The ideal solute for measuring GFR would not be protein bound, would pass freely through the filtration barrier, 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 in most animals and it meets the other criteria.

III. Functions of renal tubules are reflected by the rate of urinary excretion of a plasma substance compared to the rate of inulin excretion.
A. If excretion of a solute is greater than excretion of inulin, there is a net solute secretion by tubules.
B. 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 the filtration barrier.

IV. Functions of tubules pertaining to major solutes[1] (Fig. 8.2)

Fig. 8.2. Major physiologic processes of renal tubules that pertain to solutes and H_2O.
- Osmolality of plasma and ultrafiltrate are equal (near 300 mosmol/kg H_2O) as H_2O and nonprotein solutes pass through the glomerular filtration barrier.
- In the proximal tubules, a majority 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 proximal tubule is still near 300 mosmol/kg H_2O but the fluid volume is greatly diminished.
- In the descending limb of the loop of Henle, tubular fluid is concentrated and volume is reduced by the passive movement of H_2O. Urea may diffuse from the interstitial fluid to the tubular fluid.
- In the ascending limb of the loop of Henle, solutes (mostly Na^+, Cl^-, K^+) passively leave the tubular fluid (driven by Na^+-K^+-ATPase pump in the basolateral membrane) but H_2O remains. Thus, the tubular fluid becomes dilute and the fluid leaving the diluting segment has an osmolality near 100 mosmol/kg H_2O. Passive Ca^{2+} and Mg^{2+} resorption is dependent on an electrical gradient promoted by the Na^+-K^+-$2Cl^-$ cotransporter and the recycling of K^+; active Ca^{2+} and Mg^{2+} resorption is promoted by PTH.
- In the distal and collecting tubules, there are multiple processes involved in electrolyte balance, acid-base balance, and conservation of H_2O (see text for specifics). Major actions of ADH are to promote resorption of H_2O and urea. Major actions of aldosterone are conservation of Na^+ and Cl^- and the excretion of K^+ and H^+. The osmolality of the urine typically is > 600 mosmol/kg H_2O and may be > 2,000 mosmol/kg H_2O.
- In most healthy domestic mammals, the net function of a nephron is to excrete urea, creatinine, K^+, H^+, NH_4^+, and PO_4 and conserve Na^+, Cl^-, HCO_3^-, Ca^{2+}, Mg^{2+}, glucose, amino acids, and H_2O. The equine nephron excretes Ca^{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} values for plasma (1.028–1.034) were determined using a refractometer's urine specific gravity scale. The true specific gravity of the plasma would be nearer 1.018–1.022[62] and such a range has little to no clinical relevance.

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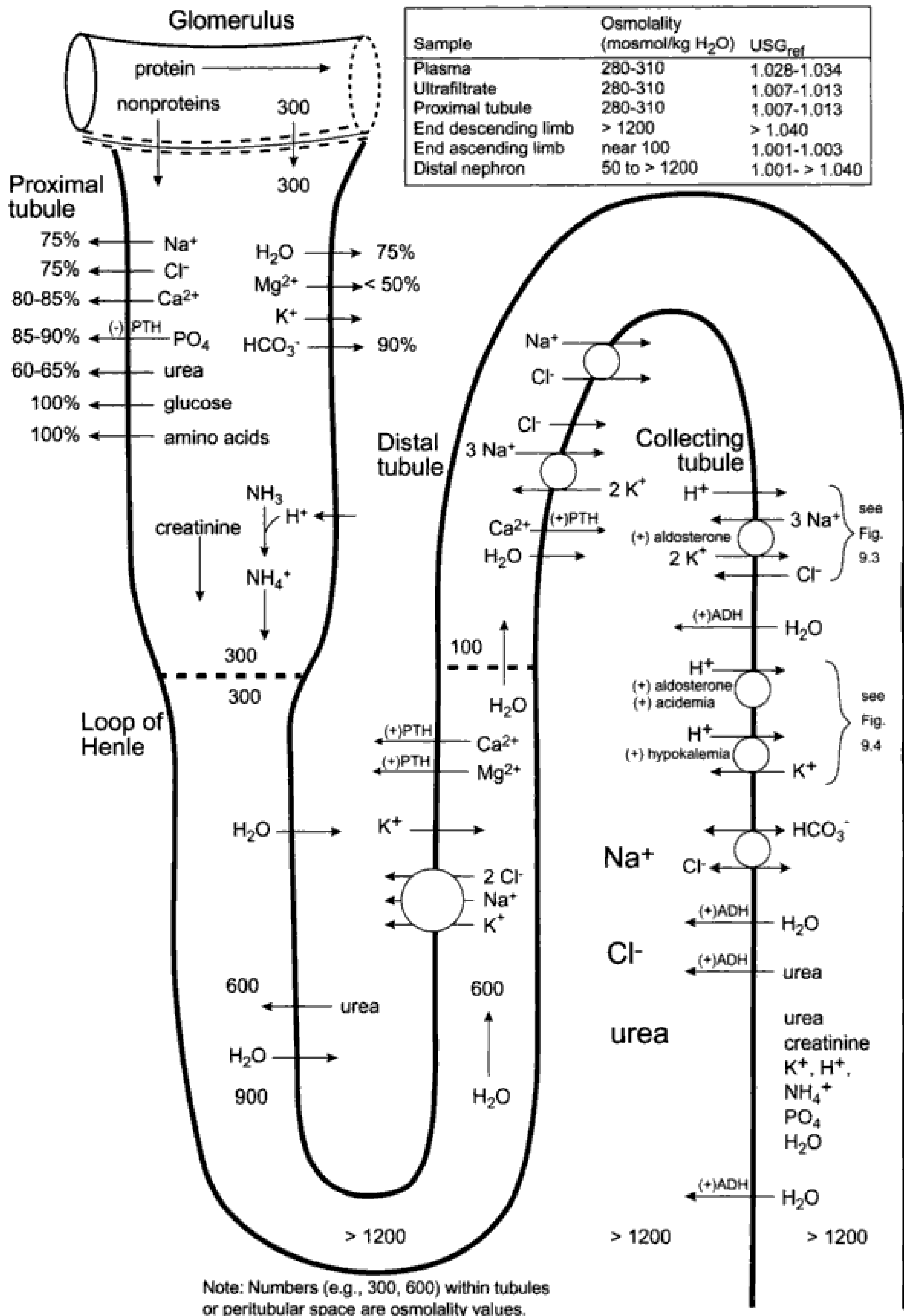

Sample	Osmolality (mosmol/kg H_2O)	USG_{ref}
Plasma	280-310	1.028-1.034
Ultrafiltrate	280-310	1.007-1.013
Proximal tubule	280-310	1.007-1.013
End descending limb	> 1200	> 1.040
End ascending limb	near 100	1.001-1.003
Distal nephron	50 to > 1200	1.001- > 1.040

Note: Numbers (e.g., 300, 600) within tubules or peritubular space are osmolality values.

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A. Na^+
1. About 75% of filtrate Na^+ is resorbed in the proximal tubules 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 phosphates. Angiotensin II stimulates the proximal tubular resorption of Na^+, Cl^-, and H_2O.
2. Na^+ is passively transported into the descending limb of the loop of Henle to maintain Na^+ in the countercurrent system.
3. Na^+ accompanies K^+ and Cl^- that is resorbed in the thick ascending limb of the loop of Henle 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.
4. ADH stimulates Na^+ and Cl^- resorption in the medullary thick limb of the loop of Henle through the Na^+-K^+-$2Cl^-$ cotransporter (a minor role for ADH).
5. Aldosterone stimulates the active resorption of Na^+ in collecting tubules 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) (see Fig. 9.3).
6. Na^+ and Cl^- resorption in the distal nephron (distal tubule and collecting duct) also involves an aldosterone-independent Na^+-Cl^- cotransporter; this process varies directly with Na^+ delivery to the distal nephron. Thiazide diuretics block this cotransporter.
7. Na^+ resorption in the distal nephron is reduced during volume expansion through the action of ANP, which reduces the number of open Na^+ channels by a guanylate cyclase pathway.

B. Cl^-
1. About 75% of filtered Cl^- is resorbed in the proximal tubules down a concentration gradient created by Na^+ and H_2O resorption and through a formate-Cl^- exchanger.
2. Cl^- is resorbed via a Na^+-K^+-$2Cl^-$ cotransporter in the thick ascending limb of the loop of Henle.
3. Cl^- is passively resorbed in the distal nephron by an electrochemical gradient established by Na^+ movement (through aldosterone and aldosterone-independent processes).

C. HCO_3^-
1. About 90% of filtered HCO_3^- is conserved indirectly in the proximal tubules during the process of H^+ secretion. H^+ secretion is mediated by the Na^+-H^+ antiporter and is dependent 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 proximal tubular 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^+-$3HCO_3^-$ cotransporter (see Fig. 9.6).
2. In the collecting ducts, HCO_3^- produced by the Type A intercalated cells enters the peritubular fluid through a Cl^--HCO_3^- exchanger (see Fig. 9.4). A reverse process in Type B intercalated cells leads to HCO_3^- secretion when there is excess HCO_3^-.

D. K^+
1. K^+ secretion occurs primarily in the principal cells of the collecting tubules and is promoted by aldosterone (see Fig. 9.3). K^+ movement from cell to tubular lumen occurs through K^+ channels opened by aldosterone. The movement is enhanced when there is a high urinary flow rate through the tubule as the K^+ is washed away and thus a concentration gradient is maintained; the movement is reduced when

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there is a low urinary flow rate through the tubule because K^+ stays longer in the tubular fluid and thus the concentration gradient is diminished.

2. ADH promotes K^+ secretion in the cortical collecting tubule, perhaps by opening K^+ channels in the luminal membrane. This process compensates for the reduced K^+ secretion that occurs with decreased urinary flow.
3. Intercalated cells conserve K^+ when there is K^+ depletion (see Fig. 9.4).

E. H^+

1. H^+ is secreted from Type A intercalated cells of the distal nephron through a H^+-ATPase pump that can work against a large concentration gradient. 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 there is K^+ depletion.
2. A limited amount of H^+ is secreted by the proximal tubular cells, after which it may be buffered by HCO_3^-, NH_3, or PO_4.

F. Ca^{2+}

1. About 80%–85% of filtrate Ca^{2+} is resorbed in proximal tubules and loops of Henle through a passive process that is dependent on Na^+ and H_2O resorption. Passive Ca^{2+} resorption occurs in the ascending limb of the loop of Henle down an electrochemical gradient established by a Na^+-K^+-$2Cl^-$ cotransporter and a recycling of K^+.
2. PTH promotes Ca^{2+} resorption through the activation of an adenylate cyclase pathway in the cortical thick ascending limb of the loop of Henle, the distal tubule, and the connecting segment between the distal tubule and the collecting ducts.
3. Vit. D promotes Ca^{2+} resorption in the distal nephron by inducing production of a calcium-binding protein. Formation of calcitriol (1–25 dihydroxycholecalciferol) is stimulated by increased PTH activity and hypophosphatemia.

G. PO_4

1. About 85%–90% of filtrate PO_4 is resorbed in the proximal tubule via the actions of a Na^+-PO_4 cotransporter.
2. The cotransporter's activity is enhanced by hypophosphatemia and insulin; it is diminished by hyperphosphatemia and increased PTH activity.

H. Mg^{2+}

1. At physiologic concentrations, nearly all of filtrate Mg^{2+} is resorbed (most in cortical thick ascending limbs of loops of Henle; also in the proximal tubules). Passive Mg^{2+} resorption is mostly down an electrochemical gradient established by the Na^+-K^+-$2Cl^-$ cotransporter and a recycling of K^+. There also may be specific transporters or channels whose activities are dependent on blood [Mg^{2+}].
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 glomerular filtration barrier, and all filtrate glucose is passively resorbed in the proximal tubules as Na^+ is resorbed by a Na^+-glucose cotransport system.
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 proximal tubules.
2. Amino acids are resorbed through carriers specific for seven amino acid groups. Small peptides are hydrolyzed at the brush border and the amino acids are resorbed.
3. Larger proteins (including albumin) enter the tubular cells through endocytosis and

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then are degraded to amino acids. In healthy dogs, some of the albumin is not resorbed and thus is present in urine in low concentrations.

K. Urea
   1. About 60%–65% of filtrate urea is resorbed in the proximal tubules down a concentration gradient created by the movement of H_2O into the cells (lowers cellular urea and raises tubular urea concentrations). 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 with urea from the interstitium entering the tubular fluid in the loop of Henle and being resorbed in the medullary aspects of the collecting ducts where it enters the medullary interstitium.
   3. Urea resorption in the distal nephron is enhanced (nearly fourfold) by ADH activity and a concentration gradient established by H_2O resorption secondary to Na^+ and Cl^- resorption. ADH increases tubular permeability to urea.
   4. 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 proximal tubules.[2,3] Crt secretion does not appear to occur in horses or cats.[4,5]
   2. In people, Crt secretion may occur through a pathway shared with organic cations. Serum [Crt] increases when other organic cations (such as cimetidine, trimethoprim, and quinidine) interfere with Crt secretion.

V. Resorption of H_2O by tubules
   A. About 30% of RPF becomes ultrafiltrate. About 75% of the ultrafiltrate H_2O is passively resorbed in the proximal tubules.
   B. H_2O is passively resorbed in the descending limbs of loops of Henle as the loop enters the hypertonic medulla.
   C. The ascending limbs of the loops of Henle and connecting segments are impermeable to H_2O.
   D. The collecting tubules are permeable to H_2O in the presence of ADH. ADH stimulates the synthesis of aquaporin-2 molecules that serve as H_2O channels from the tubular fluid through the apical membranes of the epithelial cells.

VI. Renal concentrating ability and renal diluting ability
   A. Definitions
      1. Concentrating ability: ability of kidneys to resorb filtrate H_2O in excess of filtrate solutes; ability to concentrate solutes
      2. Diluting ability: ability of kidneys to resorb filtrate solute in excess of filtrate H_2O; ability to dilute solutes
   B. Terms used to describe concentration of urine
      1. Many authors describe the solute concentration of urine with the terms *hyposthenuria*, *isosthenuria*, and *hypersthenuria* (*-sthen-* = strength). However, there is little agreement on the definitions for these terms. *Isosthenuria* should mean same (*iso-*) strength (*-sthen-*) urine (*-uria*).
      2. Our definitions (see Physical Examination of Urine, III below for correlation of USG_{ref} and urine osmolality)
         a. Isosthenuria (working definition) is the state in which urine osmolality is the

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same as plasma osmolality, whether plasma osmolality is low, normal, or high. We define "the same" as being within 100 mosmol/kg of plasma osmolality; i.e., $osmolality_u = osmolality_p \pm 100$ mosmol/kg. In most domestic mammals, such urine will have a USG_{ref} from 1.007 to 1.013, inclusively.

b. Hyposthenuria is the state in which excreted urine has an osmolality that is less than the isosthenuric values; i.e., $osmolality_u < (osmolality_p - 100$ mosmol/kg). In most hyposthenuric animals, the USG_{ref} will be less than 1.007. Such urine is dilute (the filtrate has been diluted by renal processes).

c. To be consistent with terms, eusthenuria is the excretion of urine with osmolality expected for an animal that has adequate renal function and normal hydration status, and hypersthenuria is the excretion of highly concentrated urine. However, the terms *eusthenuria* and *hypersthenuria* are rarely used.

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 allow concentration
2. Functions of tubules
   a. Proximal tubule
      (1) Approximately 30% of the plasma that enters glomeruli becomes glomerular filtrate ($USG_{ref} \approx 1.010$; osmolality $\approx$ 300 mosmol/kg H_2O).
      (2) About 60%–65% of the H_2O in the ultrafiltrate is passively resorbed by the proximal tubules (independent of body needs); the remainder passes into the loop of Henle. The solute concentration does not change much in the proximal tubule, but fluid volume decreases markedly.
   b. Descending limb of loop of Henle
      (1) 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 loop of Henle has $USG_{ref} \approx 1.010$; osmolality $\approx$ 300 mosmol/kg H_2O. Tubular fluid at the distal end of the descending limb has a high concentration ($USG_{ref} > 1.050$; osmolality > 1500 mosmol/kg H_2O).
   c. Ascending limb of loop of Henle (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 distal tubule is dilute ($USG_{ref} < 1.007$; osmolality < 200 mosmol/kg H_2O).
      (2) A functional ascending limb is necessary to maintain a hypertonic interstitial fluid in the medullary region so that passive resorption of H_2O may occur in the descending limb of the loop of Henle and in collecting tubules.
      (3) The ascending limb and the closely associated vasa recta are the major structures of the countercurrent system that maintain a hyperosmolar medullary interstitial fluid.
   d. Collecting tubules
      (1) The distal nephron is the concentrating segment 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 con-

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centrations of urea, Na^+, and Cl^- in the interstitial fluid that are produced and maintained by segmental functions of the nephron.

(3) The H_2O channels through the epithelial cells are created by membrane-associated proteins called aquaporins. When ADH binds to the renal epithelial cell membranes, it activates a secondary messenger system by which aquaporins are activated and thus membranes become permeable to H_2O. ADH also appears to increase production of aquaporins.[6]

3. For kidneys to concentrate the ultrafiltrate, 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 concentrations.
   b. Epithelial cells of the distal nephron must be responsive to ADH.
   c. There must be a concentration gradient; i.e., osmolality of interstitial fluid of the renal medulla must be greater than the osmolality of fluid in the tubules.
4. For kidneys to dilute the ultrafiltrate, the following are necessary:
   a. Active transport of Na^+ and Cl^- must occur from tubular fluid to interstitial fluid by epithelial cells of the ascending limb of the loop of Henle. This process requires delivery of Na^+ and Cl^- to the loop of Henle.
   b. Very little to no H_2O is removed from the tubular fluid by the distal nephron.

CHRONIC RENAL INSUFFICIENCY/FAILURE

I. What is chronic renal insufficiency or failure?

A. Many chronic diseases may damage kidneys sufficiently so that there is inadequate functional renal tissue to maintain health. Then the animal enters the pathophysiologic state of chronic renal insufficiency/failure.

B. There are no universally accepted definitions or criteria for staging impaired renal function. One system seems to correlate with clinical findings seen in many domestic mammals.[7,8]

1. Diminished renal reserve: 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, poor perfusion).
2. Chronic renal insufficiency: GFR is approximately 20%–50% of normal; azotemia and anemia appear; polyuria occurs because of decreased concentrating ability.
3. Chronic renal failure: GFR is < 20%–25% of normal; azotemia and impaired concentrating ability are present; kidneys cannot regulate ECF volume or electrolyte balance and thus edema, hypocalcemia (generally not in horses), and metabolic acidosis will develop; overt uremia with its neurologic, gastrointestinal and cardiovascular complications may develop.
4. End-stage renal disease: GFR is < 5% of normal; the terminal stages of uremia are present.

C. As a general concept, many authors write that more than two-thirds of functional renal mass must be lost before kidneys lose the ability to concentrate urine and more than three-fourths must be lost before an animal becomes azotemic. Such fractions help develop major concepts 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, cats became azotemic with only a 50% reduction in functional mass and most maintained renal concentrating ability (USG_{ref} > 1.040) with an estimated 83% loss in renal function.[9]

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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 processes:
      1. Damage to medullary tissues or abnormal medullary blood flow
      2. Decreased Na^+ and Cl^- transport from the ascending limb of the loop of Henle
      3. Damaged cells in the distal nephron are less responsive to ADH.

III. Polyuria in mammals with chronic renal insufficiency/failure
   A. The degree of polyuria in renal failure will not be as severe as with other diuretic states, such as diabetes insipidus, because the diminished GFR results in a decreased volume of filtered plasma H_2O. The volume of urine produced in healthy people is about 1–2 L/day, about 3–4 L/day with polyuric renal failure, and about 6–8 L/day with other polyuric states.
   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 they may retain minimal concentrating ability when they are azotemic.
   C. Progression of renal disease and loss of additional nephrons will eventually cause either oliguric or anuric renal failure.

IV. Fig. 8.3 illustrates the development of azotemia and abnormal urine volume caused by a progressive loss of nephrons.

V. Evidence of chronic renal insufficiency/failure
   A. Evidence of insufficiency/failure
      1. There is azotemia (increased [UN] and/or [Crt] in serum or plasma) due to inadequate renal ability to remove metabolic wastes from plasma.
      2. There is an inappropriately dilute USG_{ref} (often 1.007–1.013) due to a marked reduction in renal concentrating ability.
   B. Evidence of chronicity
      1. Clinical findings, including duration of signs
      2. Laboratory findings, including anemia and hypocalcemia (sometimes hypercalcemia, especially in horses)

ACUTE RENAL FAILURE

I. Acute renal failure is reversible or irreversible renal dysfunction resulting abruptly (within hours to days) from a renal disease or insult that markedly decreases GFR and leads to azotemia. Usual causes are toxicants, renal ischemia, or infections.

II. Urine volume and USG_{ref}
   A. Because of the abrupt and severe decrease in GFR, with no time for compensatory hypertrophy of healthy nephrons, the kidneys may filter little blood and produce little or no urine (oliguria or anuria).
   B. USG_{ref} can vary considerably.
      1. Urine may be unexpectedly concentrated if it was formed prior to the severe insult.

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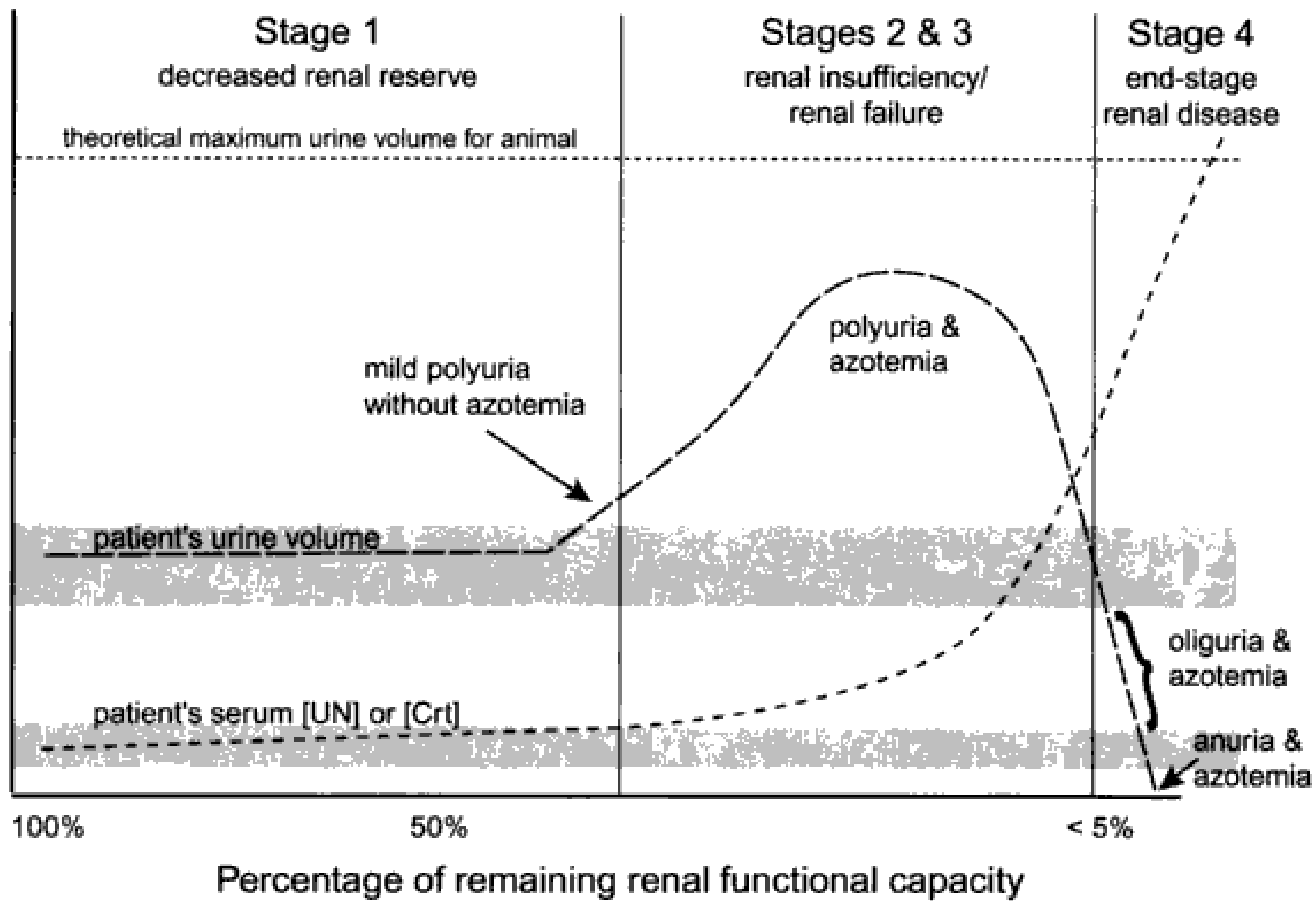

Fig. 8.3. Graphical representation of the effects of chronic renal disease and progressive dysfunction on an animal's urine volume and serum [UN] or [Crt].

Shaded gray areas represent the reference intervals for urine volume *(top bar)* and for [UN] or [Crt] *(bottom bar).* The patient's urine volume and [UN] or [Crt] concentrations 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 adequately clear urea and Crt and thus the animal is not azotemic. Also, there is sufficient ability to concentrate urine and thus polyuria is absent or not detected. As the renal dysfunction approaches Stage 2, a mild polyuria may develop because of impaired concentrating ability.
- In Stage 2 (renal insufficiency), there is sufficient loss of nephrons so that there is decreased renal concentrating ability (isosthenuria and polyuria develop) and there is insufficient excretion of urea and Crt by kidneys (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), only a few nephrons are filtering plasma and thus a marked azotemia develops. Animal also becomes oliguric or anuric because very little plasma H_2O enters the kidney. The remaining tubules are not able to concentrate the filtrate and thus USG_{ref} will reflect isosthenuria.

2. Urine may be isosthenuric because of the same mechanisms that impair concentrating ability in chronic renal disease.
3. Urine is not expected to be hyposthenuric because such a state would indicate loss of concentrating ability but retained diluting ability.

III. Acid-base status and electrolyte concentrations can become acutely abnormal.

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Table 8.2. Diseases and conditions that cause azotemia

- Decreased urinary excretion of urea or creatinine[a]
  - Prerenal diseases or conditions
    - »Hypovolemia: decreased blood volume, dehydration
    - Decreased cardiac output: cardiac insufficiency
    - »Shock
  - Renal diseases or conditions
    - »Inflammatory: glomerulonephritis, pyelonephritis, tubular-interstitial nephritis
    - »Amyloidosis
    - »Toxic nephroses: hypercalcemia, ethylene glycol, myoglobin, gentamicin, phenylbutazone
    - »Renal ischemia or hypoxia: poor renal perfusion, infarction
    - Congenital hypoplasia or aplasia
    - Hydronephrosis
    - Neoplasia (metastatic)
  - Postrenal diseases or conditions
    - »Urinary tract obstruction: urolithiasis, urethral plugs in cats, neoplasia, prostatic disease
    - »Leakage of urine from urinary tract: trauma, neoplasia
- Increased urea or creatinine production: intestinal hemorrhage, increased dietary urea or creatinine, increased protein catabolism

[a] Decreased urinary excretion involves a decreased glomerular filtration of urea or creatinine except when there is leakage of urine from urinary tract into the body.

AZOTEMIA AND UREMIA

I. Definitions
   A. Azotemia: increased nonprotein nitrogenous compounds in blood; routinely detected as increased serum [UN] and/or serum [Crt]
   B. Uremia: classically considered to mean "urinary constituents in blood"; now typically refers to the clinical signs reflecting renal failure (e.g., vomiting, diarrhea, coma, convulsions, and ammoniacal odor of breath)

II. Azotemia classifications and disorders
   A. Decreased urinary excretion of urea or Crt
      1. Prerenal azotemia: The initiating cause of abnormal urea or Crt excretion involves processes prior to the kidneys.
         a. Disorders (Table 8.2)
         b. Pathogeneses
            (1) Any process that diminishes RPF will directly cause a decreased GFR and thus decreased clearance of urea and Crt. The volume (stretch) receptors in the juxtaglomerular apparatus of the afferent arteriole "sense" reduced blood flow, which then triggers the angiotensin-renin system. Angiotensin II causes constriction of afferent and efferent glomerular arterioles, which further reduces glomerular perfusion and thus GFR.
            (2) Hypovolemia causes enhanced resorption of Na^+ and H_2O in proximal tubules, which in turn promotes passive proximal tubular resorption of urea (but not Crt) because the lower flow rate provides more time for resorption.

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(3) Hypovolemia also triggers release of ADH, which enhances resorption of urea (but not Crt) by medullary collecting tubules (aquaporin-2 allows urea transport as well as H_2O transport).

c. If decreased RPF is severe and persistent, it 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 with enough glomerular damage to cause a major decrease in GFR.
   a. Diseases or disorders (Table 8.2)
   b. Pathogeneses
      (1) Renal disease (acute or chronic) resulting in the loss of at least 65%–75% of nephron functional capacity causes reduced GFR. Reduced GFR results in inadequate renal excretion of urea and Crt from plasma (without sufficient compensation by intestinal processes) and thus serum [UN] and [Crt] increase.
      (2) Processes that contribute to a prerenal azotemia may also be present.
3. Postrenal azotemia: The initiating cause of abnormal urea or Crt metabolism is distal to the nephron.
   a. Diseases or disorders (Table 8.2)
   b. Pathogenesis of obstructive azotemia
      (1) Urinary tract obstruction causes the release of vasoactive substances (prostaglandins, angiotensin) that constrict the glomerular arterioles, thus reducing RPF and diminishing GFR; reduced GFR causes impaired clearance of urea and Crt.[10]
      (2) Impaired outflow causes a transient increase in intracapsular hydrostatic pressure that decreases GFR. The pressure diminishes with time as tubular fluid diffuses into tubular cells and less ultrafiltrate is formed.
   c. Pathogenesis of azotemia due to leakage of urine within the body
      (1) If there is leakage into the peritoneal cavity, urea and Crt enter plasma after passive absorption through peritoneal epithelium. Peritoneal [UN] equilibrates faster with plasma [UN] than peritoneal [Crt] does with plasma [Crt].
      (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 will occur. Glomerular filtration is not reduced initially, but a calculated GFR will be reduced if not all the urine produced during the test period is collected.
   d. Processes that contribute to prerenal or renal azotemia may also be present.

B. Azotemia due to increased urea production
   1. Disorders or conditions (Table 8.2)
   2. Pathogenesis
      a. Increased proteolysis results in the generation of more NH_4^+, which in turn causes increased synthesis of urea.
      b. If the rate of urea synthesis exceeds the rate of urea excretion, then serum [UN] will increase.
   3. There is usually an adequate functional renal reserve so increased urea production usually causes either mild or no azotemia in these disorders or conditions.

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Table 8.3. Major criteria used to differentiate the three types of azotemia caused by decreased GFR

Type of azotemia	Expected USG_{ref}	Urine volume	History, physical exam, or other information
Prerenal	> 1.030[a]	↓[a]	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

Note: Criteria are most applicable to dogs and generally to other mammals; USG_{ref} guidelines for cats that have concentrating ability are > 1.035 instead of > 1.030; for horses and cattle > 1.025. Prerenal, renal, and postrenal disorders may occur independently or may occur in combinations.

[a] Assuming there are not extrarenal disorders that are affecting renal concentrating ability and urine volume.

[b] Assuming there are not substances (such as protein and glucose) that interfere with USG_{ref} assessment of urine solute concentration and that plasma osmolality is not markedly increased.

[c] USG_{ref} depends on the animal's hydration status and presence or absence of concurrent renal disease. During postobstructive diuresis, the USG_{ref} values will be low and urine volume will be increased.

III. Guidelines for azotemia differentiation (Table 8.3)
   A. The cause of azotemia may be multifactorial. Both 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 both renal and extrarenal factors.
   B. The major criterion for differentiating azotemias is the USG_{ref} value. 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, USG_{ref} is expected to be > 1.030 (dogs), > 1.035 (cats), or > 1.025 (cattle, horses) because the kidneys are being stimulated to conserve H_2O.
      2. If 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 renal disease (renal azotemia) or to extrarenal disease (prerenal azotemia with impaired concentrating ability unrelated to primary renal disease).
         a. Renal disease
            (1) USG_{ref} is often 1.007 to 1.013 because of impaired tubulointerstitial function associated with nephron damage.
            (2) USG_{ref} may be greater than 1.013 but still inappropriately low if:
               (a) The renal disease impairs glomerular function more than tubulointerstitial function (especially cats or acute renal failure).
               (b) High urine concentrations of protein or glucose affect USG_{ref} such that USG_{ref} overestimates urine osmolality (see Physical Examination of Urine, III below).
               (c) Plasma osmolality is increased, so urine osmolality is greater than expected despite failure to dilute or concentrate the filtrate.

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blood. It is a clinical custom to express urea concentration in terms of nitrogen content in the urea, i.e., urea nitrogen.

a. Urea has a M_r of 60, so it weighs 60 g/mole. It is composed of 1 carbon, 1 oxygen, 2 nitrogen, and 4 hydrogen atoms. Therefore, 1 mole of urea contains 28 grams of nitrogen. Accordingly, 1 millimole of urea/dL = 60 mg of urea/dL = 28 mg of UN/dL.

b. The urea concentration, reported as the concentration of urea nitrogen, is commonly referred to as the BUN (blood urea nitrogen) concentration, although usually serum, not whole blood, is assayed. Because urea is a freely diffusable molecule for most cell membranes, the extracellular [UN] and intracellular [UN] in blood will usually be the same. Therefore, the [UN] in serum = [UN] in blood = [UN] in plasma.

2. Unit conversion

a. mg/dL of UN × 0.3570 = mmol/L of urea (SI unit, nearest 0.5 mmol/L)[11]

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 for 1 day at room temperature, several days at 4°–6°C, and at least 2–3 months frozen.[12]

C. Principles of urea assays

1. Vitros dry reagent slide on Vitros instrument

a. In a reaction catalyzed by urease, urea is hydrolyzed to form NH_3 and CO_2. The NH_3 reacts with an indicator to generate a colored dye, which is detected by reflectance spectrophotometry.

b. Positive interference

(1) NH_4^+: Free NH_4^+ in the sample will react with the dye. Because [NH_4^+] in most plasma and serum samples is < 1% of urea concentration, the degree of interference should be clinically insignificant as long as the sample is not contaminated with NH_4^+ (e.g., quaternary ammonium compounds).

(2) Hemolysis: Hemoglobin at 50 mg/dL will cause an increased [UN] of about 1 mg/dL.

2. Boehringer Mannheim wet reagents on Hitachi instrument: In a reaction catalyzed by urease, urea is hydrolyzed to form NH_4^+ and CO_2. The NH_4^+ reacts in a coupled reaction that results in the consumption of NADH and thus a decreased absorbance that is measured by kinetic spectrophotometry.

III. Increased [UN] in serum or plasma (azotemia) (Table 8.2)

IV. Decreased [UN] in serum or plasma (Table 8.4)

Table 8.4. Diseases and conditions that cause decreased serum or plasma [UN]

Disorders that cause decreased urea synthesis
*Hepatic insufficiency: hepatocellular disease, portosystemic shunts
Urea cycle enzyme deficiencies (very rare)
Disorders that cause increased renal excretion of urea
*Disorders that cause impaired proximal tubular resorption of urea: glucosuria
Central or renal diabetes insipidus

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A. Disorders that cause decreased urea synthesis
  1. Hepatic insufficiency
    a. Extensive hepatocellular disease that results in marked reduction in functional hepatic mass (> 80% loss) and thus sufficient decrease in urea synthesis to cause a decreased [UN] and a corresponding increase in [NH_4^+]
    b. Portosystemic shunt (congenital or acquired)
      (1) Less NH_4^+ is delivered to hepatocytes from the intestines.
      (2) There is less uptake of NH_4^+ by hepatocytes because of decreased functional hepatic mass due to atrophy, necrosis, or fibrosis.
  2. Urea cycle enzyme deficiencies (congenital, extremely rare)
B. Disorders that cause increased renal excretion of urea
  1. When less H_2O is resorbed in the proximal tubules (e.g., due to glucosuria or expanded extracellular volume), less of the filtered urea is resorbed in the proximal tubules because the resorption of H_2O creates the concentration gradient for urea resorption.
  2. In central and nephrogenic diabetes insipidus, reduced ADH activity or response in the medullary collecting tubules results in decreased resorption of both urea and H_2O.
C. 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 concerning Crt (Fig. 8.5)

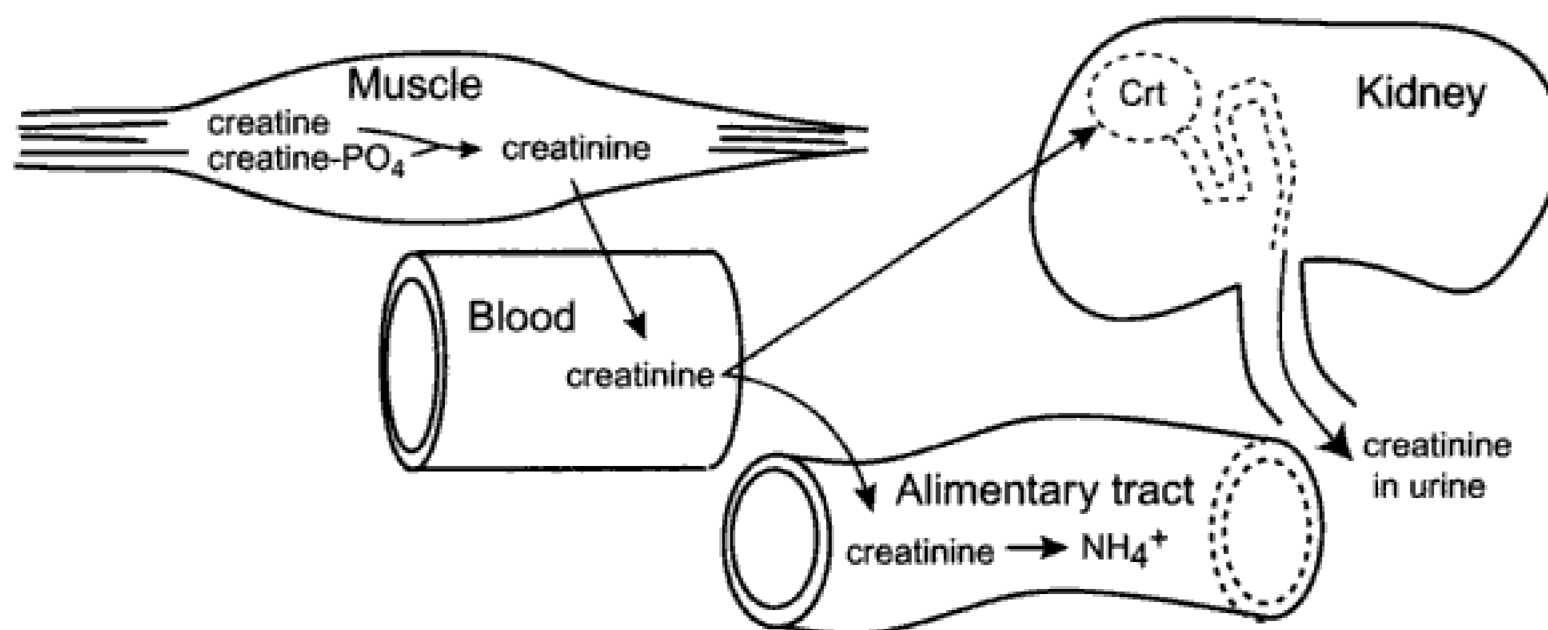

Fig. 8.5. Physiologic processes or concepts concerning Crt.

Crt is the product of creatine (not creatinine) degradation. Creatine phosphate serves as a high-energy molecule for muscle contractions (creatine + ATP ⇔ creatine-PO_4 + ADP). Crt enters plasma after the degradation of creatine or creatine-PO_4 in muscle fibers (animal's muscle or dietary meat). Crt is excreted from the body via kidneys and intestine.

- Crt passes freely across the glomerular filtration barrier; it is not resorbed by tubules. Small quantities may be secreted by proximal tubules when there is increased plasma [Crt].
- Crt is also excreted or degraded in feces in people[64,65] and in saliva of cattle.[63] Alimentary tract excretion is suspected to occur in dogs, cats, and horses, since Crt is diffusable across most cell membranes.

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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, nearest 10 µmol/L)[11]
  B. Sample
    1. Serum or plasma may be used in most spectrophotometric assays.
    2. Crt is stable at 4°C for 1 day and is more stable when frozen.[12]
  C. Principles of Crt assays
    1. Vitros dry reagent slide on Vitros instrument: Crt is hydrolyzed to creatine, which then enters a series of reactions that results in H_2O_2 reacting with an indicator to generate a colored dye that is detected by reflectance spectrophotometry.
    2. Boehringer-Mannheim wet reagents on Hitachi instrument: Crt reacts with picric acid to form a colored complex; the rate of formation of the colored complex is measured by a spectrophotometer.
    3. In older assays, non-Crt chromogens (e.g., proteins, glucose, acetone, and acetoacetate reacted to produce Crt-like chromogens) caused a positive interference in Crt assays. Conversion to kinetic assays has reduced the interference.

III. Increased [Crt] in serum or plasma (azotemia) (Table 8.2)
  A. Increased [Crt] is typically due to a pathologic process associated with decreased GFR (prerenal, renal, or postrenal processes). Potentially, increased Crt production and release from damaged myocytes (e.g., exertional rhabdomyolysis) could contribute to increased serum [Crt], but Crt is quickly cleared from plasma if there is adequate renal function.
  B. Baseline serum [Crt] may vary among individuals because of variations in total body muscle mass or meat intake, but these factors are not expected to cause azotemia.

IV. Decreased [Crt] in serum or plasma
  A. Not clinically recognized or clinically significant
  B. In most species, serum [Crt] is near the analytical sensitivity of Crt assays and thus 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.
  B. In theory, [Crt] is a better indicator than [UN] of decreased GFR because the quantity of Crt presented to the kidneys is more constant and it is not resorbed by the tubules, whereas urea is resorbed. Factors that may affect [Crt] and [UN] include:
    1. Hypovolemia causes increased 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 distal nephron.
    2. Increased protein in intestinal contents (high protein diet, massive intestinal hemorrhage) leads to increased generation of NH_4^+ and subsequently urea if digestive and absorptive processes are functioning.
    3. Intestinal excretion of urea and Crt may influence serum [UN] and [Crt].

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Table 8.5. $(UN{:}Crt)_s$ ratios in azotemic dogs and cats

	Prerenal		Renal		Postrenal	
No. of cases	6		78		17	
	Average	Range	Average	Range	Average	Range
[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
$(UN{:}Crt)_s$ ratio	55	10–260	30	7–102	31	12–128

Source: Adapted from Finco DR, Duncan JR. 1976. Evaluation of blood urea nitrogen and serum creatinine concentrations as indicators of renal dysfunction: A study of 111 cases and a review of related literature. J Am Vet Med Assoc 168:593-601.

II. Serum UN:Crt ratio
   A. Clinical observations have suggested that a serum UN:Crt ratio can help differentiate prerenal and renal azotemias, and the following statements have been made:
      1. An increased serum [UN] but a normal serum [Crt] is most likely a prerenal azotemia.
      2. If serum [Crt] is increased proportionately more than serum [UN], then the azotemia is probably renal or postrenal.
   B. Published conclusions and data from azotemic dogs[13] (Table 8.5)
      1. It is not possible to reliably differentiate renal from extrarenal azotemias by means of the serum UN:Crt ratio.
      2. [UN] and [Crt] in serum should be regarded as crude indices of renal function; both lack diagnostic sensitivity and specificity for renal dysfunction due to renal disease.

CREATININE (Crt) CLEARANCE RATE

I. Crt clearance rate is the rate Crt is cleared from plasma by the kidneys; it is a good estimate of GFR in domestic animals, but it is not equivalent to GFR. A decreased Crt 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. Crt clearance rate formula (Eq. 8.1)

$$\text{Creatinine clearance rate} = \frac{[\text{Crt}]_u}{[\text{Crt}]_s} \times \text{volume}_u \div \text{time} \div \text{bw} \qquad \textbf{(8.1.)}$$

$[\text{Crt}]_u$ = Crt concentration in collected urine during a timed collection period

$[\text{Crt}]_s$ = Crt concentration in serum from a blood sample 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 creatinine/min/kg

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III. Composition of urine 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 plasma presented to kidneys
   B. Renal functions, including filtration, secretion, and absorption
   C. Material (chemicals, cells) added to glomerular filtrate as it flows through kidneys, ureters, urinary bladder, urethra, and prepuce or vagina/vulva

IV. It is important to know the method of urine collection (i.e., voided, cystocentesis, catheterization, or "off-floor") when you are interpreting UA results. Results expected in healthy dogs, cats, horses, and cattle are provided in Table 8.6.

Table 8.6. Expected UA results in healthy dogs, cats, horses, and cattle

	Dog	Cat	Horse	Cattle
Physical				
Color	yellow[a]	yellow[a]	yellow[a]	yellow[a]
Clarity	clear	clear	hazy-turbid	clear
USG_{ref}	1.015-1.045[b]	1.015-1.065[b]	1.020-1.050[b]	1.025-1.045[b]
Chemical				
pH	6.0-7.5	6.0-7.5	7.5-8.5	7.5-8.5
Protein	neg – 1+[c]	neg	neg	neg
Glucose	neg	neg	neg	neg
Ketone	neg	neg	neg	neg
Heme	neg	neg	neg	neg
Bilirubin	neg – 1+[c]	neg	neg	neg
Urobilinogen	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)[d]				
WBC /hpf	< 5	< 5	< 5	< 5
RBC /hpf	< 5	< 5	< 5	< 5
Bacteria /hpf	none	none	none	none
Casts /lpf	none[e]	none[e]	none[e]	none[e]
Epithelial cells /lpf	none to few[f]	none to few[f]	none to few[f]	none to few[f]
Crystals /lpf	none[g]	none[g]	none[g]	none[g]
Other	—	—	mucus	—

[a] Intensity of yellow will typically vary proportionately with USG_{ref}.
[b] Assuming normal hydration status and no treatments that alter resorption of H_2O by kidneys.
[c] Trace and 1+ reactions should be found in the more concentrated samples.
[d] The number of cells or structures seen per microscopic field will differ when the diameter of the viewed field differs because of differences between microscope objective and ocular lens.
[e] A few hyaline casts usually are not associated with a pathologic state; occasional granular casts can be found in healthy animals.
[f] Varies with method of collection; large round epithelial cells and squamous cells are expected in voided and some catheterized samples.
[g] Phosphate crystals in dogs and cats and carbonate crystals in horses and cattle are common examples of crystals whose presence may not indicate a pathologic state; see Table 8.10.

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PHYSICAL EXAMINATION OF URINE

I. Urine color (pigments)
   A. Physiologic processes
      1. 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 dilute or less concentrated than dark yellow urine, but not always (Plate 6.*A*).
   B. Analytical concepts: Gross assessment of urine color is typically done on fresh, well-mixed urine.
   C. Abnormal urine color (pigmenturia)
      1. Abnormal color indicates the presence of abnormal pigments in the urine. Other parts of the urinalysis or other assays are needed to determine which pigment or pigments are present.
      2. Common abnormal colors and the substances that create them (concurrent pigmenturias may alter expected colors)
         a. Red: erythrocytes, hemoglobin, myoglobin
         b. Red-brown: erythrocytes, hemoglobin, myoglobin, methemoglobin
         c. Brown to black: methemoglobin from Hgb or myoglobin
         d. Yellow-orange: bilirubin
         e. Yellow-green or yellow-brown: bilirubin, biliverdin
      3. Horse urine may turn red or brown during storage or when exposed to snow. The pigmenturia is reported to be due to pyrocatechin (pyrocatechol),[28] which is the aromatic portion of catecholamines. A pathologic state is not associated with this pigmenturia.

II. Urine clarity
   A. Physiologic processes
      1. Expect clear but may have mild turbidity due to suspended particles (e.g., epithelial cells, crystals)
      2. Equine urine is frequently turbid or cloudy due to 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.
   C. Cloudiness or turbidity indicates the presence of formed elements, such as cells, crystals, bacteria, and casts, in the urine.

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, NH_4^+) and metabolic products (urea, 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.
   B. Analytical concepts
      1. Specific gravity (also called relative density), a physical property of a solution, is the

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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. Specific gravity is a unitless ratio.

2. Refractive index as an estimate of urine specific gravity (USG_{ref})
   a. The refractive index of urine is measured with a refractometer and it is used as a routine clinical estimation of urine specific gravity (USG_{ref}). The refractive index 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 are bent (refracted). As solute is added to H_2O, the degree to which the light slows down 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 is correlated 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. Most temperature-compensated refractometers have USG_{ref} scales that are calibrated for the normal composition of human urine (Fig. 8.6). However, there are refractometers that are calibrated for constituents of canine, feline, and large animal urine (Fig. 8.7).

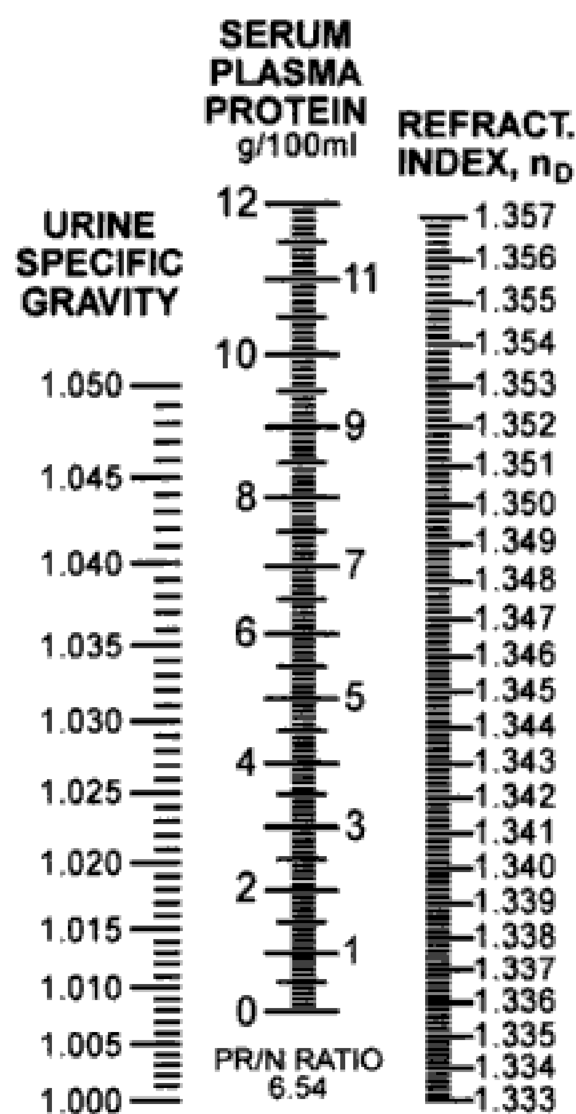

Fig. 8.6. Illustration of scales in a Leica TS400 handheld refractometer.
The refractometer measures the fluid's refractive index, and the "urine specific gravity" and "serum plasma protein" scales are used to estimate USG_{ref} values and total protein concentrations, respectively. The refractometer scales were calibrated for human samples. (Image used with permission from Leica Microsystems Inc., Buffalo, N.Y.)

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2. Healthy animals with normal or adequate renal function can excrete urine with a broad USG_{ref} range, depending on what the kidneys are being challenged to do.
   a. Maximal urine dilution in domestic mammals as assessed by USG_{ref}: near 1.001
   b. Maximal urine concentration as assessed by USG_{ref}: cats > 1.080, dogs near 1.060, horses and cattle near 1.050
   c. Usual USG_{ref} values when H_2O intake is adequate and hydration status is normal: dogs (1.015–1.045); cats (1.015–1.065); horses (1.020–1.050); cattle (1.025–1.045). However, USG_{ref} may be lower or higher in animals with normal renal function.

D. Interpretation of USG_{ref} values
1. A USG_{ref} value 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, however, it is difficult to formulate firm guidelines for the interpretation of USG_{ref} values in all cases.
2. Animals with impaired renal concentrating ability will have one or more of the following defects.
   a. ADH deficiency is present (central diabetes insipidus).
   b. Epithelial cells of distal nephrons are not responsive to ADH (nephrogenic diabetes insipidus).
   c. Solute overload was present (too much solute entering the loop as occurs with osmotic diuresis, renal failure, or increased GFR), resulting in a high flow rate and decreased resorption of tubular H_2O.
   d. Decreased medullary hypertonicity
      (1) Prolonged hyponatremia or hypochloremia
      (2) Defective Na^+ and Cl^- transport in loop (e.g., loop diuretics)
      (3) Decreased urea production due to liver disease
      (4) Solute overload or prolonged diuresis
3. General guidelines for USG_{ref} interpretation are in Table 8.7.
4. USG_{ref} values in various disorders or conditions
   a. USG_{ref} > 1.030 in an oliguric dog, > 1.035 in an oliguric cat, > 1.025 in oliguric horses or cows
      (1) Nonrenal processes (e.g., hypovolemia, 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 tubules, thus concentrating the tubular fluid and thus urine.
   b. USG_{ref} < 1.030 in an obviously dehydrated dog, < 1.035 in a dehydrated cat, < 1.020 in a dehydrated cow or horse
      (1) Such findings indicate a renal concentrating defect that could be due to renal or extrarenal disease.
      (2) Pathogeneses of the specific causes vary with the pathologic states (see polyuric disorders below).
   c. USG_{ref} = 1.020–1.035 in a polyuric animal
      (1) Diabetes mellitus: Glucosuria causes an osmotic diuresis by inhibiting the passive resorption of H_2O in the proximal tubules. If it persists, the high flow rate may result in impaired resorption of Na^+, Cl^-, and urea, and thus medullary tonicity decreases.
      (2) Potentially seen with renal glucosuria

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release; thus, there might be reduced ADH activity (conversely, hypovolemia is probably stimulating ADH release).

(6) 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.

(7) Liver failure
   (a) Increased NH_4^+ excretion may inhibit renal concentrating mechanisms.
   (b) Decreased urea synthesis may lead to a decreased medullary urea concentration, thus a decreased medullary concentration gradient (medullary washout).

(8) Hypokalemia
   (a) Hypokalemia makes collecting tubules less responsive to ADH, perhaps due to reduced generation of AMP[37] or aquaporin.[6]
   (b) K^+ is needed for Na^+ and Cl^- resorption in the ascending limb of the loop of Henle and thus hypokalemia may impair countercurrent function.[1,38]

(9) Hypoparathyroidism: The pathogenesis is not well understood.

(10) Feline hyperthyroidism: The pathogenesis is not well understood.

(11) Psychogenic polydipsia: Excessive H_2O consumption leads to expanded ECF volume and hypoosmolality; polyuria results from increased GFR and decreased ADH secretion.

(12) Others: diuretic therapy, alcohol administration, IV fluid administration, dextrose or mannitol fluid therapy

(13) Thyroiditis: Occasional dogs with thyroiditis (but not hypothyroidism) have polyuria and polydipsia. The pathogenesis is not known; it may not be directly related to thyroiditis.[39]

CHEMICAL EXAMINATION OF URINE (QUALITATIVE OR SEMIQUANTITATIVE)

I. Major concepts

A. Semiquantitative results of urinalysis procedures (chemical and microscopic) are used to detect or characterize pathologic states. The concentration of a solute in urine will depend on two major factors: (1) amount of solute excreted in the urine over time (may come from reproductive tract) and (2) amount of H_2O excreted by the urinary system during the same time interval.

1. The semiquantitative results of the reagent pad systems are graded on scales provided by the manufacturers of the reagent strips (Table 8.8).
   a. A 1+ result indicates there was enough of the solute present to give a 1+ reaction but not enough to give a 2+ reaction.
   b. When reactions are read by visual examination, the distinction 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.
2. Relationship of urine solute concentration, urine volume, and daily urinary excretion of solutes (Eq. 8.3)

$$\text{Daily urinary solute excretion} = [\text{solute}]_u \times \text{urine volume / day} \qquad \textbf{(8.3.)}$$

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Table 8.8. Semiquantitative values or terms of solute concentrations estimated by urinalysis reagent strip reactions (Multistix® by Bayer)

	Glucose (mg/dL)	Bilirubin	Ketone (mg/dL)	Heme[a]	Protein (mg/dL)	Urobil. (mg/dL)
Negative[b]	< 75–125	< 0.4–0.8	< 5–10	< 0.015–0.062	15–30	—
Trace	100	—	5	—	10	0.2–1.0
1+	250	small	15	small	30	2
2+	500	moderate	40	moderate	100	4
3+	1000	large	80	large	300	8
4+	2000	—	160	—	1000	—

Note: Reagent strip has a pH indicator pad with a range of 5.0–8.5 units that are read to the nearest 0.5 units by color comparison (except some strips do not have a 5.5 reaction).

[a] Heme test is commonly referred to as either the "blood" or "occult blood" test.

[b]Reported ranges are the reported analytical sensitivity limits of the reactions in contrived urine; reactions may vary in actual samples. Ketone sensitivity measured with acetic acid. Heme sensitivity measured with Hgb. Protein sensitivity measured with albumin. All units in "negative" row are mg/dL.

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/day to 200 mL/day), then the urinary excretion of the solute doubled from one day to the next (from 150 mg/day to 300 mg/day).
b. If the urine solute concentration doubled (from 150 to 300 mg/dL) from one day to the next but the urine volume remained constant (100 mL/day), then the urinary excretion of the solute doubled from one day to the next (from 150 mg/day to 300 mg/day).
c. If the urine solute concentration doubled (from 150 to 300 mg/dL) from one day to the next but the urine volume halved (from 100 mL/day to 50 mL/day), then the urinary excretion of the solute remained constant from one day to the next (150 mg/day).

3. 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 urinary excretion of solutes remains constant (e.g., 1 g/day) but 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 urinary excretion of solutes remains constant (e.g., 1 g/day) but 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).
4. Use of concepts to interpret urinalysis results
   a. Dog #1 with a urine glucose concentration 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 concentration of 1.0 g/dL and a USG_{ref} of 1.030.
   b. Dog #3 with a urine protein concentration of 50 mg/dL and a USG_{ref} of 1.040 is probably not proteinuric (healthy dogs may have urine protein concentrations of 4–65 mg/dL; USG_{ref} is typically 1.020–1.045). However, Dog #4 with a urine protein concentration of 50 mg/dL and a USG_{ref} of 1.010 is proteinuric.

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Table 8.9. Major disorders or conditions that cause abnormal chemistry results in a routine UA

- Aciduria
  - »Expected in healthy carnivores, omnivores, suckling herbivores, and herbivores on milk diet
  - »Acidoses, some metabolic and potentially with respiratory
  - »Associated with hypochloremic metabolic alkalosis (paradoxical aciduria)
  - Hypokalemia
  - H^+ production by bacteria
  - Proximal tubular acidosis (if HCO_3^--depleted)
- Alkalinuria
  - »Expected in healthy herbivores and after meals in monogastric mammals (alkaline tide)
  - »Urea degradation: spontaneous in older samples, initiated by urease-containing bacteria
  - Alkaloses, some metabolic and potentially with respiratory
  - Proximal tubular acidosis (early)
- Proteinuria
  - Prerenal (overflow): hemoglobinuria, myoglobinuria, paraproteinuria
  - »Glomerular: glomerulonephritis or amyloidosis
  - Tubular: congenital or acquired proximal tubular diseases
  - »Hemorrhagic or inflammatory proteinuria
  - False positive reaction (see text)
- Glucosuria (glycosuria)
  - »Hyperglycemia
  - Renal: congenital or acquired proximal tubular diseases
  - False positive reaction (see text)
- Ketonuria
  - »Ketosis
  - False positive reaction (see text)
- Heme positive[a]
  - »Hematuria (pathologic, iatrogenic, estral)
  - »Hemoglobinuria
  - Myoglobinuria
  - False positive reaction (see text)
- Bilirubinuria
  - »Expected in concentrated urine of healthy dogs
  - »Hemolytic diseases
  - »Hepatobiliary diseases
  - False positive reaction (see text)

[a] In most reagent systems, the heme test [illegible] called either the "blood" test or the "occult blood" test although the assay is designed to detect heme.

or it may result from other factors (secondary hyperaldosteronism or enhanced secretion of Na^+ and H^+ by the ascending limb because the Na^+-K^+-$2Cl^-$ cotransporter is blocked by furosemide).[1]

6. Proximal renal tubular acidosis (if HCO_3^--depleted): A decreased conservation of HCO_3^- by proximal tubules allows HCO_3^- to buffer more H^+ in tubular fluid and thus there is a higher urine pH than expected in an acidotic animal (may be alkalinuric). However, when plasma $[HCO_3^-]$ decreases, the remaining tubular function

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may be enough to conserve the filtered HCO_3^-. Then, there will not be sufficient HCO_3^- in the tubular fluid to buffer the H^+ and aciduria may be present.

D. Alkalinuria: suggests decreased excretion of H^+ (Table 8.9)
   1. Urea splitting or hydrolysis: Breakdown of urea releases two $-NH_2$ groups that each quickly accept H^+ ions to form NH_4^+; removal of free H^+ from urine makes the urine more alkaline.
      a. May occur with delayed completion of urinalysis
      b. May be caused by urease-containing bacteria (e.g., *Staphylococcus* and *Proteus*), either *in vivo* or *in vitro*
   2. Respiratory alkalosis: Probably less H^+ is secreted by the distal nephron because of less stimulation of the H^+-ATPase pump.
   3. Distal renal tubular acidosis: Decreased H^+ excretion by the distal nephron can lead to an inappropriately high urine pH (> 6.0) in the face of acidosis; the pH may not be alkaline.
   4. Proximal renal tubular acidosis(see explanation in preceding aciduria section).

III. Protein in urine

A. Physiologic processes
   1. Many small proteins (usually M_r < 68,000) can pass through the glomerular filtration barrier. In most healthy animals, the proteins are resorbed in the proximal tubules and thus very little to no protein is detected in urine samples.
   2. Urine of healthy dogs may contain measurable protein concentrations without evidence of urinary tract disease.
      a. Most of the protein is albumin.
      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; 4–65 mg/dL with a CBB method; 4–95 mg/dL with a trichloroacetic acid method.[41]
   3. Tamm-Horsfall protein is mucoprotein that apparently is secreted by the loop of Henle and part of the distal tubule and collecting ducts; it is soluble above pH 7 but insoluble below pH 7. It is a major component of hyaline casts, and it may be part of the matrix of granular casts.

B. Analytical concepts
   1. Reagent strip method
      a. Principle: The reagent pad contains a colorimetric pH indicator (tetrabromphenol blue) at acidic pH; negatively charged proteins bind the dye and change the pad's color.
      b. Changes in color of the pad correspond to estimated protein concentrations (Table 8.8).
      c. Abnormal urine color (pigmenturia) may interfere with reagent pad color and therefore with estimation of protein concentration.
      d. Falsely increased readings may occur in highly buffered alkaline urine[42] or in urine that contains quaternary ammonium salts or chlorhexidine.
      e. Analytical sensitivity and specificity
         (1) Detects albumin better than globulins, which are less negatively charged. Protein in cells (e.g., epithelial cells and leukocytes) reacts very poorly with reagents.
         (2) Protein concentrations needed to give a trace to 1+ reaction: albumin (14–21

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mg/dL), α-globulin (20–30 mg/dL), β-globulin (40–50 mg/dL), γ-globulin (> 1000 mg/dL), light chains (26–52 mg/dL), and hemoglobin (5–50 mg/dL)[43,44]

2. SSA turbidity
   a. Principle: Proteins are denatured by acids and form a precipitate that is seen as increased solution turbidity.
   b. Results may be expressed on a visual turbidity scale (1+ to 4+) or visually compared against standard solutions to interpolate concentrations. There are also spectrophotometric SSA methods that give more quantitative results.
   c. SSA reacts with albumin better than globulins (reportedly 2–4 times as well) and will detect Bence Jones proteins (concentration-dependent).
   d. Falsely increased readings can be caused by X-ray contrast media, tolbutamide, penicillin (massive dose), sulfisoxazole, tolmetin sodium, and turbidity caused by co-precipitation of crystals because of the low pH of SSA.
   e. Falsely decreased readings can be caused by highly buffered alkaline urine.[26,45]
3. TCA method
   a. Principle: Proteins are denatured by acids and form a precipitate that is seen as increased solution turbidity.
   b. The TCA method is less affected by the albumin:globulin ratio in the sample than is the SSA method. The TCA method is temperature sensitive and needs to be completed at 20°–25°C.[46]
4. CBB assay
   a. Principle: The amount of dye binding to amine groups of amino acids is proportional to the quantity of protein present.
   b. Quantitative spectrophotometric assays are not part of routine UA but may be used to document urine protein concentration. These assays are minimally affected by albumin:globulin ratios in the urine and they will detect Bence Jones proteins.
5. Benzethonium chloride assay
   a. Principle: Benzethonium chloride reacts with proteins to produce a turbidity that is proportional to the amount of protein present.
   b. This quantitative turbidimetric assay is reported to react similarily with albumin and γ-globulins. Hemoglobin will react with the reagent.

C. Proteinuria (Table 8.9) (Fig. 8.9)
1. Prerenal (overflow, overload, preglomerular) proteinuria
   a. A pathologic state causes an increased plasma concentration of a small protein that passes through the glomerular filtration barrier. If the amount of filtered protein exceeds the ability of proximal tubules to resorb it, the protein is excreted in the urine. Examples: paraproteinuria (light chains: monomer M_r = 23,000, dimer M_r = 46,000), hemoglobinuria (dimer M_r = 34,000), myoglobinuria (M_r = 17,000), and postcolostral proteinuria in food animals.
   b. Light chain, hemoglobin, and myoglobin molecules are detected by routine urine protein assays.
   c. Overflow proteinurias do not produce hypoproteinemia.
2. Glomerular proteinuria
   a. Glomerular disease causes damage to the filtration barrier and a decrease in selective permeability. The glomerulus becomes increasingly permeable to larger proteins or to negatively charged proteins.

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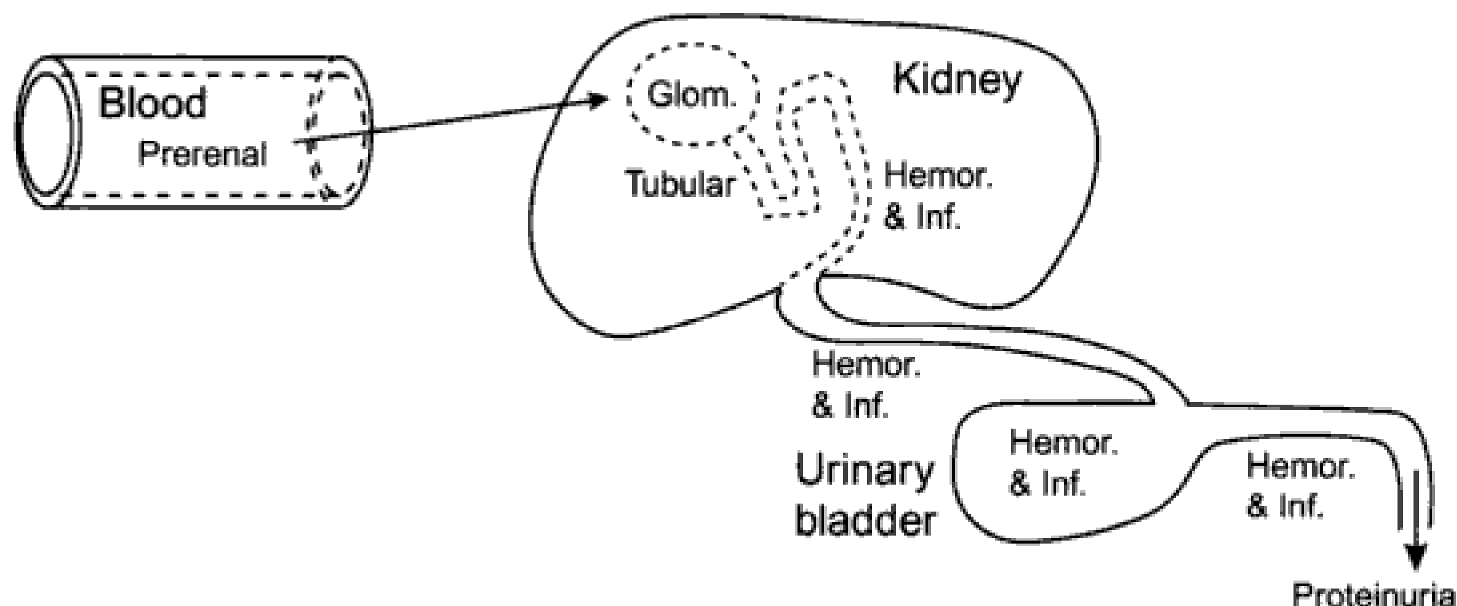

Fig. 8.9. Four major types of proteinuria.

- In prerenal proteinurias (Prerenal), small proteins (e.g., Hgb dimers, light chains, myoglobin) present in plasma at increased concentrations are excreted in urine because they pass through the glomerular filtration barrier and are incompletely resorbed in tubules.
- In glomerular proteinurias (Glom.), glomerular disease causes damage to the filtration barrier and a decrease in selective permeability. Glomeruli become increasingly permeable to larger or negatively charged plasma proteins. These proteins pass through the defective filtration barrier and are incompletely resorbed by tubules, so they are excreted in urine.
- In tubular proteinurias (Tubular), proximal renal tubules are defective so that proteins that normally are resorbed from ultrafiltrate (e.g., some albumin and smaller globulins) are not, and thus they are excreted in the urine.
- In hemorrhagic and inflammatory proteinurias (Hemor. & Inf.), plasma proteins or hemoglobin enter the urine because of hemorrhage or inflammation involving renal tubules, renal pelvis, ureters, urinary bladder, urethra, or genital tract tissues.

   b. Albuminuria may be the first evidence of glomerular disease, but with greater damage, other proteins, including larger globulins, appear in the urine. Prolonged mild or rapid severe glomerular proteinuria will cause a selective hypoproteinemia (loss of plasma proteins except for the largest forms) (see Chap. 7).
   c. In people, a transient proteinuria occurs after exercise and it is considered a form of glomerular proteinuria.
3. Tubular proteinuria
   a. Proximal renal tubules are defective so that proteins that normally are resorbed from ultrafiltrate (e.g., some albumin and smaller globulins) are not, and thus they are excreted in the urine.
   b. Tubular proteinurias are usually associated with acute renal diseases (toxicoses, hypoxia) but can be congenital; they do not produce hypoalbuminemia.
4. Hemorrhagic or inflammatory proteinuria (also called secretory or postrenal proteinuria, but hemorrhage and exudation may occur in kidneys)
   a. Hemorrhagic: hemorrhage into the genitourinary tract due to impaired hemostasis, including blood vessel damage by inflammation, trauma, neoplasia, or other necrosis
   b. Inflammatory: exudation of plasma proteins through vessel walls into the genitourinary tract due to inflammation
   c. The postrenal proteinurias are the most common proteinurias. The quantity of protein lost is usually not sufficient to cause hypoalbuminemia, but there may be

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mild hypoalbuminemia due to inflammation or massive hemorrhage. Proteins detected in the urine originate primarily from plasma; proteins from cells (leukocytes, epithelial cells) are poorly detected by the urine protein assays.

d. Evidence to support this type of proteinuria is usually found in other urinalysis results: inflammatory (pyuria), hemorrhagic (hematuria).

e. Must consider reproductive tract sources (e.g., prostatitis, estral bleeding), especially in voided urine samples.

D. Protein-losing nephropathy and renal failure concepts (Fig. 8.10)

1. Urea and Crt are small molecules that freely pass through the glomerular filtration barrier (sieves). Some filtrate urea is resorbed by tubules. The remaining urea and Crt are excreted in urine and thus do not accumulate in blood. Except for small amounts in dogs, albumin does not pass through the glomerular filtration barrier and thus it remains in the 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 glomeruli. If the ability to resorb proteins is exceeded, then a proteinuria will be present. The continual loss of protein will lead to hypoalbuminemia. As long as there is an adequate number of functional glomeruli, urea and Crt will be adequately removed from blood and azotemia will 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 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.

IV. Glucose in urine

A. Physiologic processes

1. Glucose is a relatively small molecule that passes freely through the glomerular filtration barrier and enters the ultrafiltrate.

2. Glucose is resorbed passively in the proximal tubules via a Na^+-glucose cotransport system that is dependent on a favorable gradient established by active Na^+ transport from tubular cells to the peritubular fluid.

3. Renal tubular transport maximum for glucose varies between domestic mammals; approximate transport maximums are 180–220 mg/dL in dogs,[47] about 290 mg/dL in cats,[47] about 150 mg/dL in horses[48] and calves,[49] and probably lower in mature cattle.

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; H_2O_2 reacts with an indicator to give a color change in the reagent pad. The degree of color change is proportional to the glucose concentration (Table 8.8).

b. Falsely increased reactions may be caused by H_2O_2 and sodium hypochlorite.

c. Falsely decreased reactions may be caused by ascorbic acid, ketones, very concentrated urine samples, and possibly cold urine.

2. Copper-reduction method (Clinitest)

a. Principle: Cu^{2+} reacts with a reducing substance (e.g., hexoses) to produce cuprous (Cu^+) oxide and cuprous hydroxide and thus a color change.

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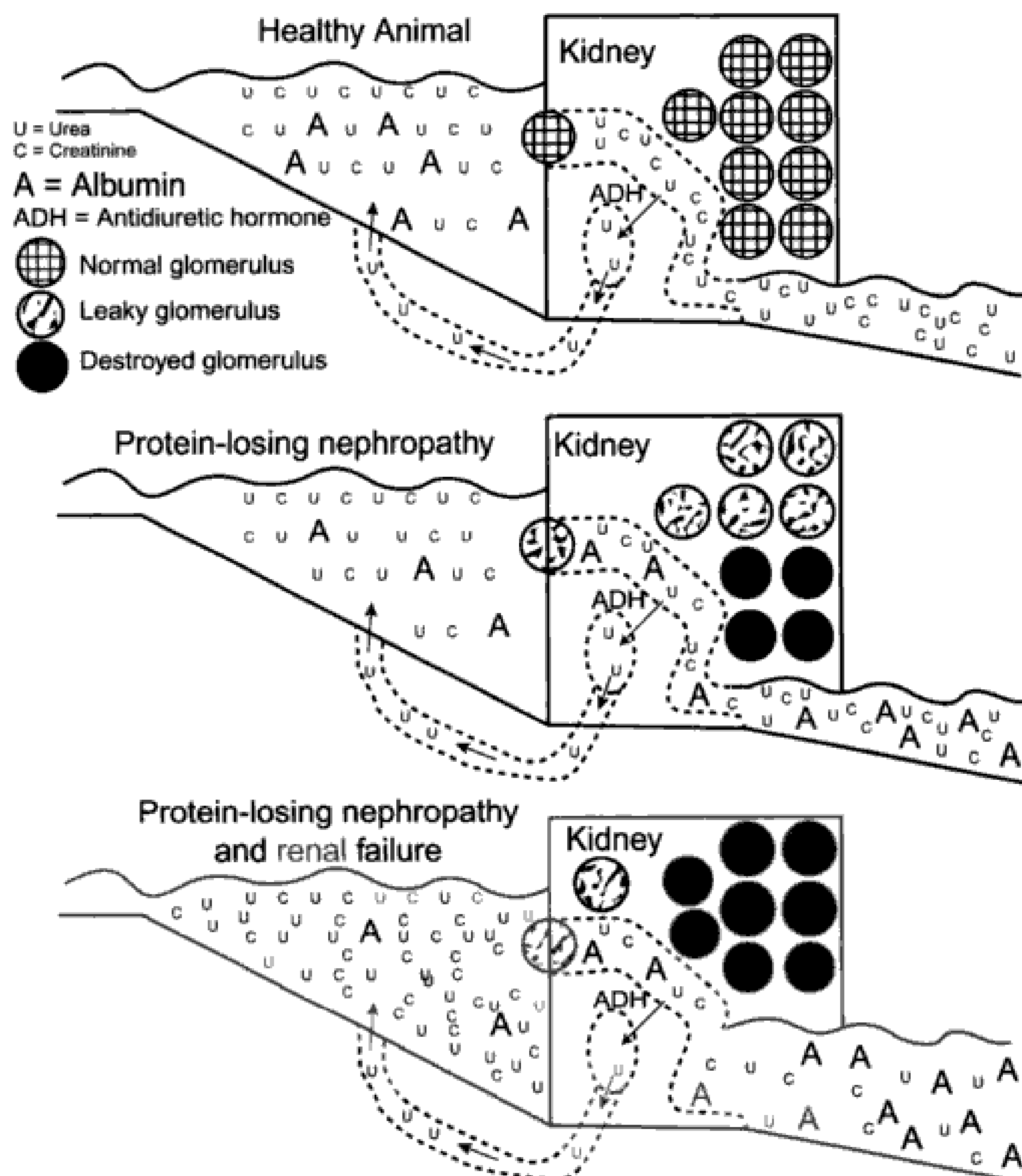

Fig. 8.10. Protein-losing nephropathy and renal failure.

Illustrations depict a body's ECF as a lake, kidneys as a dam, and the urine as the river below the dam. Urea and creatinine molecules are small fish in the lake; 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 is reclaimed to maintain ECF H_2O and urea content. Only 1 of 10 nephrons is shown as a filter with a connected tubular system.

- In the healthy animal, urea and creatinine freely pass 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 and thus 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 creatinine removed from the lake and thus azotemia does not develop.
- In the 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 a hypoalbuminemia and proteinuria. The remaining filters are insufficient to keep urea and creatinine removed from the lake and thus azotemia develops. Also, the remaining nephrons are not able to adequately conserve H_2O and thus polyuria develops.

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b. Semiquantitative results for standard method: negative, ≈ 250, ≈ 500, ≈ 750, ≈ 1000, ≈ 2000 mg/dL (probably more accurate than reagent strip method)
c. False positive reactions may be caused by cephalosporin and ascorbic acid.

C. Glucosuria (glycosuria) disorders (Table 8.9)
1. Hyperglycemic glucosuria
a. Transient or persistent hyperglycemia results in more glucose in the ultrafiltrate than can be resorbed by proximal tubules.
b. Hyperglycemia is typically concurrent with the glucosuria, but a transient hyperglycemia and delay in bladder emptying may mask concurrence.
2. Renal glucosuria (normoglycemic glucosuria)
a. Transient or persistent glucosuria results from defective resorption of glucose caused by damaged or abnormal proximal tubules.
b. Tubular abnormalities can be acquired or congenital
(1) Acquired: proximal renal tubular toxicosis or ischemia
(2) Congenital: Fanconi syndrome and pure primary renal glucosuria (basenji, Norwegian elkhound, Shetland sheepdog)[50]
3. Glucose in tubular fluid will cause osmotic diuresis (H_2O is "held" by the glucose in the tubular fluid, especially in the proximal tubules) and thus 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. Acetoacetic acid and β-hydroxybutyric acid are ketoacids that are produced by hepatocytes but dissociate at physiologic pH values to their anionic form and H^+.
2. Ketone bodies are not expected in the urine of healthy mammals that have adequate intake of nutrients.
3. Ketone bodies may enter urine by both glomerular filtration of plasma and by tubular secretion. The tubular secretion process probably shares a transport process with other organic anions. After entering the tubular fluid, acetoacetate and β-hydroxybutyrate are nonresorbable.[1]

B. Analytical concepts
1. Reagent strip method (Bayer)
a. Principle: Acetoacetate (mostly) and acetone (less reactive) form colored complexes with nitroprusside; the amount of color change reflects the amount of ketones present (Table 8.8). The reagent system does not react with β-hydroxybutyrate, the ketone body that 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. Trace reactions may occur in urine with high specific gravity and low pH.[51]
2. Acetest tablet method (Bayer)
a. Principle: same as reagent strip method; easier to detect color change and thus considered a more sensitive method
b. Method may be used as a qualitative assay for blood, plasma, urine, and milk.

C. Ketonuria (Table 8.9)
1. Ketonuria occurs when there is increased mobilization of lipids because of a shift in energy production from carbohydrates to lipids (e.g., diabetes mellitus, starvation,

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heme reaction.[45] Erythrocytes should be microscopically identified in the urine sediment to confirm hematuria; erythrocytes tend to lyse if urine is very alkaline or unconcentrated (e.g., USG_{ref} < 1.015), so they may not be seen on examination of the sediment.

2. Hemoglobinuria
    a. During clinical intravascular hemolysis, plasma hemoglobin dimers pass through the glomerular filtration barriers and enter the ultrafiltrate. If they are not completely resorbed by 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 (see Table 4.8); hemoglobinuria may also be created by lysis of erythrocytes after they enter urine; in such cases, hemoglobinemia should not be present (unless erythrocytes were lysed during blood collection or handling).
3. Myoglobinuria
    a. Myocyte necrosis or damage allows the release of myoglobin from the cell into the interstitial fluid, lymph, and finally blood, from which the small protein easily passes into the glomerular filtrate. If it is not completely resorbed by 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 exertional or paralytic rhabdomyolysis of horses (azoturia).

VII. Bilirubin in urine

A. Physiologic processes
1. Bilirubin is not expected in urine of domestic mammals other than the dog (see below).
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 regurgitated to plasma, Bc passes freely through the glomerular filtration barrier and is excreted in urine.
3. Renal tubular cells in at least some species (e.g., dogs) have the ability to convert heme (from resorbed hemoglobin of hemoglobinuria) to Bu and then 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 is not filtered by most glomeruli. However, because healthy dogs may have mild albuminuria and glomerular diseases can cause proteinuria, Bu bound to albumin may be present in urine.

B. Analytical concepts
1. Reagent strip method
    a. Principle: Bilirubin becomes coupled with diazotized dicholoroaniline in a strongly acid medium; the coupling produces color changes that reflect the amount of bilirubin present (Table 8.8).
    b. Falsely increased reactions may be caused by indican and metabolites of etodolac.
    c. Falsely decreased reactions may be caused by ascorbic acid.
2. Ictotest method: Although based on the same analytical principle, it is considered to have higher analytical sensitivity.
3. Bilirubin molecules are degraded to biliverdin by exposure to ultraviolet light. Before

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C. Pyuria: increased leukocyte concentration (number/hpf) in urine sediment (Table 8.10)
   1. Urinary tract inflammation
      a. Inflammation of mucosal or submucosal tissues or renal parenchyma may allow leukocytes (mostly neutrophils and macrophages) to migrate from blood to urine. Inflammation can be caused by infections (bacteria, fungi, parasites) or noninfectious processes (neoplasia, urolithiasis, necrosis).
      b. The method of sample collection (voided, cystocentesis, or catheterization) may help define the site of the inflammatory process. That is, leukocytes 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 localize the inflammation; e.g., pollakiuria and stranguria would suggest lower urinary tract involvement; fever, neutrophilia, or azotemia would suggest nephritis.
   2. Genital tract inflammation
      a. Leukocytes may enter urine from the male or female genital tract before (e.g., prostate) or during (e.g., preputial or vaginal source) micturition. The chance of contamination during micturition is reduced if the sample is collected midway through micturition.
      b. The inflammatory process may or may not be of clinical significance (e.g., mild posthitis).

III. Erythrocytes in urine sediment (Plate 6.*C*)
   A. Physiologic processes: A few erythrocytes (< 5/hpf) may be found in urine from healthy mammals.
   B. Analytical concepts
      1. Enumerated as range or mean of erythrocytes seen in most 400× fields (hpf)
      2. Erythrocytes may crenate in urine and can be confused with leukocytes in nonstained sediment.
      3. Erythrocytes may lyse in urine either before or after urine is collected; lysis tends to occur in unconcentrated or mildly concentrated urine (USG_{ref} < 1.015) or very alkaline urine, especially if there is a delay in analysis.
      4. If erythrocytes are seen in the urine sediment, the heme reaction should be positive.
   C. Hematuria: increased erythrocyte concentration (number/hpf) in urine sediment (Table 8.10)
      1. Pathologic hemorrhage
         a. Vascular damage due to 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)
      2. Iatrogenic hemorrhage
         a. Blood vessels may be damaged during bladder palpation, cystocentesis, or catheterization.
         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

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IV. Bacteria in urine sediment (Plate 6.*D* and *E*)

A. Physiologic processes: Urine formed by kidneys should be sterile. Low numbers of bacteria may gain access to urine via the distal urinary tract or genital tissues.

B. Analytical concepts

1. Enumerated as a relative density of bacteria seen in most 400× fields (hpf)
2. Bacteria (especially cocci) may be difficult or impossible to differentiate from small particulate debris by routine sediment examination.
3. If bacteria are of clinical significance, pyuria is expected but not always found.
4. Bacteria may multiply in urine after it is collected.
5. There is no standard method of reporting the concentration of bacteria in urine sediment. Bacterial numbers may be graded (few to many) or bacteria may simply be reported as present or absent. Routine centrifugation of urine does not appreciably concentrate bacteria in the sediment because they do not "spin down."[53] This is in contrast to other suspended particulates such as cells.

C. Bacteriuria (Table 8.10)

1. Determining clinical significance of bacteriuria involves consideration of the presence or absence of pyuria, source of sample, concentration of bacteria, and other evidence of a urinary tract infection.
2. 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 rods/mL or 100,000 cocci/mL may be required for detection by routine urinalysis but as few as 1000/mL may be significant in a cystocentesis sample. The cutoff value (based on quantitative culture) between significant bacteriuria and contamination depends on the species of animal and the urine collection method used.[54]

V. Casts in urine sediment (Plate 6.*F*, *G*, and *H*)

A. Physiologic processes

1. Casts are cylindrical concretions formed in renal tubular lumens from proteins, intact cells, or cellular debris. Most are thought to have a matrix composed of Tamm-Horsfall mucoproteins secreted by epithelial cells of the loops of Henle, distal tubules, and collecting ducts.
2. A few 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 (< 2/lpf) but a shower of casts may occur after physical activity.

B. Analytical concepts

1. Enumerated as a range or mean of casts seen in most 100× fields (lpf)
2. Casts are classified by their appearance, which reflects their content: fine and coarse granular (cell debris, plasma protein), epithelial cell (tubular epithelial cells), erythrocyte, leukocyte, fatty (lipid droplets or lipid accumulation within deteriorated cells), waxy (perhaps deterioration of granular cast), mixed (combinations of previously mentioned).
3. Casts deteriorate in urine (especially alkaline urine) and thus casts are best detected in fresh urine samples.
4. Casts may be pigmented by bilirubin, hemoglobin, or myoglobin.

C. Cylindruria (casts in urine) (Table 8.10)

1. Hyaline cast may be found in healthy animals and occur more commonly in animals with glomerular proteinurias.

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2. Granular, cellular, or fatty casts typically reflect active tubular degeneration or necrosis; they may be the first evidence of toxic nephrosis (e.g., gentamicin-induced, or hemoglobinuric and myoglobinuric nephropathies) or renal ischemia. A few granular casts can be found in the urine of healthy mammals.
3. Leukocyte or erythrocyte casts reflect inflammation or hemorrhage, respectively, involving renal tubules.
4. Waxy casts are uncommon and seen primarily with chronic renal disease.
5. Hemoglobin and myoglobin casts are red- to brown-pigmented, granular casts and may be seen with hemoglobinuria or myoglobinuria.

D. Pathogenesis (prevailing theory)
1. Hyaline casts form from the conglutination of Tamm-Horsfall mucoprotein that is secreted by tubular cells of the loop of Henle, distal tubules, and collecting ducts. The reason for the conglutination is not understood.
2. Granular, lipid, and cellular casts form when cellular debris or cells are trapped in the Tamm-Horsfall mucoprotein. Granular casts may also form from the incorporation of plasma proteins into the Tamm-Horsfall mucoprotein matrix. Waxy casts form from the deterioration and solidification of granular casts.
3. Casts may 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 the possibility of active renal tubular disease.

VI. Epithelial cells in urine sediment (Plate 6.*I* and *J*)

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 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; squamous epithelial cells lining the distal urethra appear round or polygonal.

B. Analytical concepts
1. Enumerated as range or mean of epithelial cells seen in most 100× fields (lpf)
2. Superficial squamous epithelial cells are difficult or impossible to differentiate from the occasional transitional epithelial cells that have angular borders.
3. Round transitional epithelial cells are difficult or impossible to differentiate from the round cells that occur in the intermediate layers of squamous epithelium.
4. In nonstained sediment, individual renal 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.
5. All cells deteriorate in urine and thus are best examined in fresh urine.

C. Clinical significance of epithelial cells in urine
1. May be found in healthy animals, especially in catheterized samples
2. More transitional epithelial cells may slough from inflamed or hyperplastic mucosa.
3. Neoplastic epithelial cells may be detected in urine sediment. Differentiation of neoplastic, hyperplastic, and dysplastic cells is best accomplished in stained centrifuged preparations made from very fresh, newly formed urine. However, differentiation may be difficult. Dysplastic and hyperplastic cells are common in urine from animals with cystitis.

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$$\text{Amount of analyte excreted / day} = \text{analyte concentration} \times \text{urine volume / day} \quad \textbf{(8.4.)}$$
$$\text{Example: analyte concentration} = 50\ \text{mg / dL; urine volume / day} = 300\ \text{mL / day}$$
$$\text{Amount of analyte excreted / day} = 50\ \text{mg / dL} \times 300\ \text{mL / day} = 150\ \text{mg / day}$$

C. Because a 24-hr excretion study is difficult, more convenient but sometimes less accurate methods to assess urinary excretion are used, such as urine:plasma ratios or analyte:Crt ratios.

III. Analyte urine:plasma ratios
A. Theory
1. The rate of urinary excretion or clearance of a substance can be calculated using an excretion rate formula (Eq. 8.5.a).

$$\text{Analyte excretion rate} = \frac{[\text{Analyte}]_u}{[\text{Analyte}]_p} \times \text{volume}_u \div \text{time} \div \text{bw} \quad \textbf{(8.5.a.)}$$

$$\text{Analyte excretion rate} \propto \frac{[\text{Analyte}]_u}{[\text{Analyte}]_p} \times \text{constant} \quad \textbf{(8.5.b.)}$$

2. If urine volume, time, and body weight are considered constants, then the urine:plasma ratio is proportional to the rate of urinary excretion of a substance (Eq. 8.5.b.). Those factors are not constants, but the ratio does tend to reflect the urinary excretion of a substance (analyte) or solutes.

B. Analyte urine:plasma ratios for differentiation of prerenal azotemia and renal azotemia
1. Osm_u:Osm_p ratio reflects the kidneys' ability to conserve H_2O.
   a. A high ratio reflects an ability to concentrate solutes. An animal with prerenal azotemia due to dehydration should have a high ratio.
   b. A ratio near 1.0 indicates isosthenuria and thus failure to concentrate or dilute the ultrafiltrate solutes. In an animal with renal azotemia, the ratio should be near 1.0.
2. UN_u:UN_p and Crt_u:Crt_p ratios assess 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 ratios for horses illustrate the application of the ratios (also called urinary indices) (Table 8.11). The ratios would vary between species but the general concepts apply across species.

IV. $(\text{Prot:Crt})_u$ ratio
A. Theory
1. Increased protein loss via the urinary system is best determined by a 24-hr protein excretion study, i.e., determining mg of protein lost/day/kg 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 health. In addition, if urinary Crt clearance is decreased, then the rate of glomerular protein loss should be decreased because Crt more easily passes through the glomerular filtration barrier than do protein molecules. However, if more protein enters urine

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Table 8.11. 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 (mosm/kg)	727–1456	458–961	226–495
$\text{Osm}_u : \text{Osm}_p$	2.5–5.2	1.7–3.4	0.8–1.7
$[\text{UN}]_u : [\text{UN}]_p$	34–100	15–44	2.1–14
$[\text{Crt}]_u : [\text{Crt}]_p$	2–344	51–242	2.6–37
F.E. of Na^+	0.01–0.7	0.02–0.5	0.8–10.1

Source: Adapted from Grossman BS, Brobst DF, Kramer JW, Bayly WM, Reed SM. 1982. Urinary indices for differentiation of prerenal azotemia and renal azotemia in horses. J Am Vet Med Assoc 180:284-288.

through damaged glomeruli or through other processes, then the rate of protein excretion compared to Crt excretion will be increased.

3. Considering the above concepts, comparing rate of urinary protein loss to Crt excretion should reflect true changes in protein loss via the urinary system. This concept can be seen in the comparison of urinary excretion formulas and the derivation of the $(\text{Prot:Crt})_u$ ratio(Eq. 8.6).

$$\frac{\text{Protein excretion rate}}{\text{Creatinine excretion rate}} = \frac{\frac{[\text{Prot}]_u}{[\text{Prot}]_s} \times \text{volume}_u \div \text{time} \div \text{bw}}{\frac{[\text{Crt}]_u}{[\text{Crt}]_s} \times \text{volume}_u \div \text{time} \div \text{bw}} \quad \textbf{(8.6)}$$

For a randomly collected urine sample, some factors in the numerator and denominator formulas are either the same or remain relatively constant.

- The urine volume, the time the urine formed, and the body weight values are the same in both formulas.
- The serum [Prot] and [Crt] probably remained constant during the time the collected urine was formed.

Therefore, the relative rate of protein excretion compared to creatinine excretion can be estimated as follows.

$$\frac{\text{Protein excretion rate}}{\text{Creatinine excretion rate}} = \frac{\frac{[\text{Prot}]_u}{\text{constant}}}{\frac{[\text{Crt}]_u}{\text{constant}}} \quad or \quad \frac{\text{Protein excretion rate}}{\text{Creatinine excretion rate}} \propto \frac{[\text{Prot}]_u}{[\text{Crt}]_u} = (\text{Prot:Crt})_u \text{ ratio}$$

B. Published data
   1. When dogs with potential nonrenal proteinuria were excluded, there was good correlation between the $(\text{Prot:Crt})_u$ ratio and the quantity of urinary protein excreted per day (either mg/day or mg/kg/day).[56-58] Results of three studies are compared in Table 8.12.
   2. Interpretation guidelines for the $(\text{Prot:Crt})_u$ ratio based on these studies
      a. Healthy dogs: $(\text{Prot:Crt})_u$ ratio < 0.5
      b. Borderline values: $(\text{Prot:Crt})_u$ ratio $= 0.5–1.0$

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Table 8.12. (Prot:Crt)$_u$ ratios and 24-hr urinary protein excretion studies

	Group	White et al.[ab]	Grauer et al.[bc]	Center et al.[ab]
(Prot:Crt)$_u$ 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-hr urinary protein	Healthy	1.9–11.7 (8)	0.6–5.1 (16)	0.2–7.7 (19)
excretion (mg/kg)	Proteinuric	32.2–271.1 (10)	12.2–287.5 (14)	7.5–533.7 (38)[f]

Sources: White JV, Olivier NB, Reimann K, Johnson C. 1984. Use of protein-to-creatinine ratio in a single urine specimen for quantitative estimation of canine proteinuria. J Am Vet Med Assoc 185:882-885; Grauer GF, Thomas CB, Eicker SW. 1985. Estimation of quantitative proteinuria in the dog, using the urine protein-to-creatinine ratio from a random, voided sample. Am J Vet Res 46:2116-2119; Center SA, Wilkinson E, Smith CA, Erb H, Lewis RM. 1985. 24-hour urine protein/creatinine ratio in dogs with protein-losing nephropathies. J Am Vet Med Assoc 187:820-824.

[a] Protein method: trichloroacetic acid protein method.

[b] Creatinine method: alkaline picric acid.

[c] Protein method: Coomassie brilliant blue.

[d] Number in parentheses is the number of dogs in the 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 a glomerulonephritis and its (Prot:Crt)$_u$ ratio was increased (0.47).

c. Dogs with glomerular proteinuria: (Prot:Crt)$_u$ ratio > 1.0. (Note: Other forms of proteinuria could give similar results but they were excluded from the cited studies.)

3. (Prot:Crt)$_u$ ratios were not influenced by collection period (day or night) or gender of animal.[59]

C. Diagnostic significance of increased (Prot:Crt)$_u$ ratio
1. (Prot:Crt)$_u$ ratio should be increased in any animal with proteinuria, including prerenal (overflow), glomerular, tubular, and inflammatory/hemorrhagic proteinurias. Other clinical information is used to differentiate the proteinurias (see Chemical examination of urine, III above).
2. Glomerular proteinurias tend to be more severe and cause hypoalbuminemia or hypoproteinemia. However, earlier stages of glomerular damage may cause only mild proteinuria.

D. Estimated (Prot:Crt)$_u$ ratio
1. A semiquantitative method of estimating a (Prot:Crt)$_u$ ratio is available (Petstix® 8 reagent strips).
2. Until critical studies are available that assess the predictive values of the system with veterinary samples, the method probably should be considered to be another subjective assessment of urinary protein excretion and similar to interpreting the relationship between urine protein concentration and USG_{ref}.

E. Comparison of urine protein concentration to urine [Crt] reduces the variability due to the amount of H_2O excreted by kidneys. If all else is equal, renal conservation of H_2O will cause a proportional increase in urine protein and Crt concentrations. Similar information could be obtained for less expense by dividing the urine protein concentration by a factor derived from USG_{ref} (such as the last two digits or USG_{ref} - 1).

V. Fractional excretion (F.E.) ratios or percentages

A. Theory
1. In a random urine sample, the F.E. of substance X will reflect the relative rate of uri-

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nary excretion of substance X compared to Crt. If substance X passes freely through the glomerular filtration barrier and is neither secreted nor resorbed (like Crt), the F.E. would be 1.0. If some of substance X were resorbed after freely entering the filtrate, the F.E. would be < 1.0. Therefore, for solutes that freely pass the glomerular filtration barrier (e.g., electrolytes), F.E. 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 F.E. formula (Eq. 8.7), substance X is Na^+.

$$\frac{\text{Urinary Na}^+\text{ excretion rate}}{\text{Urinary creatinine excretion rate}} = \frac{\frac{[Na^+]_u}{[Na^+]_s} \times \text{volume}_u \div \text{time} \div \text{bw}}{\frac{[Crt]_u}{[Crt]_s} \times \text{volume}_u \div \text{time} \div \text{bw}} \quad \textbf{(8.7.)}$$

For a randomly collected urine sample:

- Urine volume, time the urine formed, and the body weight values are the same in both formulas and thus cancel out.
- Serum $[Na^+]$ and [Crt] will probably be nearly the same but can vary.
- Urine $[Na^+]$ and [Crt] are expected to vary because of multiple factors.

Considering these factors, the relative rates of Na^+ and Crt urinary excretion can be expressed as follows:

$$\frac{\text{Urinary Na}^+\text{ excretion}}{\text{Urinary creatinine excretion}} = \frac{\frac{[Na^+]_u}{[Na^+]_s}}{\frac{[Crt]_u}{[Crt]_s}} = \frac{[Na^+]_u}{[Na^+]_s} \times \frac{[Crt]_s}{[Crt]_u}$$

$$\text{Therefore, fractional excretion of Na}^+ = \text{F.E. Na}^+ = \frac{[Na^+]_u}{[Na^+]_s} \times \frac{[Crt]_s}{[Crt]_u}$$

$$\text{Or expressed as a percentage, percent excretion of Na}^+ = \frac{[Na^+]_u}{[Na^+]_s} \times \frac{[Crt]_s}{[Crt]_u} \times 100$$

2. A major advantage of the F.E. study over a 24-hr excretion study is that the assessment can be done on a random single urine sample. However, the urine and serum (or plasma) samples for the assessment should be collected near the same time.

B. Interpretive concepts
   1. An F.E. ratio provides the relative rate of excretion of an analyte compared to Crt. For a F.E. ratio to accurately reflect the 24-hr urinary excretion of an analyte, Crt excretion needs to be WRI and relatively constant.
   2. An increased F.E. ratio may reflect increased urinary excretion of an analyte.
      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. Decreased F.E. ratio of an analyte may result from the opposite processes.

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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 F.E. ratio may also occur with a decreased Crt excretion.
a. An increased F.E. of K^+ would occur if the renal excretion of K^+ is maintained through secretion of K^+ but Crt excretion is decreased.
b. An increased F.E. of PO_4 may be due to decreased GFR (thus less filtration of Crt and PO_4) and decreased tubular resorption of filtered PO_4 because of increased PTH activity.

C. Clinical uses of fractional excretion studies
1. F.E. of Na^+
a. In a hyponatremic animal, an increased F.E. of Na^+ indicates that renal excretion of Na^+ is contributing to the hyponatremia. Such a process may reflect decreased aldosterone activity, increased ANP, or renal tubular disease.
b. In a hyponatremic animal, a decreased F.E. of Na^+ indicates that extrarenal factors are causing the hyponatremia and the kidneys are attempting to conserve Na^+ through the actions of aldosterone, angiotensin II, or ADH.
c. F.E. of Na^+ increases in renal failure and decreases with prerenal azotemia, while GFR is decreased in both conditions.
2. F.E. of PO_4
a. In a hypocalcemic animal, an increased F.E. of PO_4 suggests increased PTH activity as may occur with nutritional or renal secondary hyperparathyroidism.
b. In a hypocalcemic animal, a decreased F.E. of PO_4 suggests that decreased PTH activity may be contributing to the hypocalcemic state.
c. In a patient with hypercalcemia due to primary hyperparathyroidism, the F.E. of PO_4 is increased.
3. F.E. of GGT
a. GGT is part of renal tubular cell membranes and thus more GGT is excreted in urine when there is renal tubular cell damage. GGT activity in a random urine sample may or may not be increased with renal tubular disease. GGT activity should reflect the GGT concentration in the urine, which is determined by relative amounts of GGT and H_2O in the urine. Thus, dilute urine would be expected to have lower GGT activity than concentrated urine.
b. An increased F.E. of GGT indicates active renal tubular damage or necrosis; it does not determine if there is or is not renal insufficiency/failure, and it is not a reflection of GFR.[60]

D. F.E. studies are not commonly used in veterinary medicine because information obtained may not be required for diagnosis and case management. Also, appropriate reference intervals are difficult to obtain for each species, especially when one considers that separate reference intervals may be needed for different assay systems and different diets.

H_2O DEPRIVATION AND ANTIDIURETIC HORMONE (ADH) RESPONSE TESTS IN ANIMALS WITH POLYURIA/POLYDIPSIA (PU/PD)

I. Renal concentrating ability is assessed in a routine urinalysis by determining the USG_{ref}. Typically, the USG_{ref} value along with other case information (historical and physical find-

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ings, other laboratory data) allows the veterinarian to identify the probable causes of a PU/PD disorder (see Table 8.7). 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 the challenges vary, the following provides the major general concepts. Reference articles or texts should be consulted for specific interpretation guidelines.

II. Abrupt H_2O deprivation test[14]
   A. General concepts
      1. The basic purpose is to assess the ability of the kidneys to concentrate the ultrafiltrate by inducing an abrupt stimulus (hypovolemia or hyperosmolality) for renal H_2O retention. The test may be used to evaluate the undiagnosed PU/PD patient that consistently has 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 procedure
      1. Baseline information is collected and may include body weight, USG_{ref}, urine and serum osmolality, or serum [Na^+].
      2. Access to H_2O is abruptly removed, animal is monitored, and findings are compared with baseline information until findings indicate one of the following:
         a. Animal becomes dangerously dehydrated.
         b. Kidneys can concentrate urine.
         c. Animal was adequately challenged and its kidneys did not adequately concentrate urine.
   C. Basic interpretations
      1. If the animal demonstrates ability to concentrate urine (exceeds USG_{ref} or urine osmolality criteria), the PU/PD state is a primary PD disorder.
      2. If the animal does not demonstrate ability to concentrate urine, several possibilities exist.
         a. Procedure had to be halted before renal concentrating mechanisms produced concentrated urine.
         b. Secondary medullary hypotonicity (medullary washout) may be present due to persistent polyuria from a variety of disorders.
         c. A disorder that causes nephrogenic diabetes insipidus is present.
         d. Central diabetes insipidus is present.

III. Gradual H_2O deprivation test[14]
   A. 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 washout from prolonged PU is expected or if there is failure to concentrate after abrupt H_2O deprivation. The gradual decrease in H_2O intake will allow the kidneys to re-establish a medullary concentration gradient. It tends to be a less dangerous procedure in severely PU animals but animals still must be thoroughly monitored for development of dehydration.
   B. The major difference between the gradual and the abrupt procedures is that H_2O intake and urine output are measured and recorded over 2–3 or more days. Then, H_2O avail-

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ability is decreased by 10% each day. Like 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 re-establishment of medullary hypertonicity. The major disadvantage is the time requirement; it may take over a week to complete the test.

IV. ADH (vasopressin) response test[14]

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 cause urine concentration.

B. Basics of procedure

1. H_2O access is *ad libitum* during the study.
2. After administration of aqueous vasopressin or DDAVP, renal concentrating ability is assessed by measuring USG_{ref} or urine osmolality.

C. Expected findings

1. Central diabetes insipidus: Kidneys did not concentrate urine during H_2O deprivation but did when stimulated by exogenous ADH.
2. Nephrogenic diabetes insipidus: Kidneys did not concentrate urine during H_2O deprivation or after stimulation by exogenous ADH; same results may occur with "medullary washout" due to any primary PU or PD disorder.

V. Modified H_2O deprivation test[61]

A. Combination of H_2O deprivation test and ADH response test

B. Procedure

1. Abruptly deprive animal of H_2O, then measure urine osmolality hourly until there is less than 5% increase in urine osmolality between consecutive hourly samples.
2. ADH administered and urine collected 1 hr postinjection

C. Expected results

1. Healthy dogs: urine osmolality after H_2O deprivation > 1000 mosmol/kg; 0% increase in urine osmolality after ADH administration
2. Partial central diabetes insipidus and partial renal diabetes insipidus cases (including dogs with hyperadrenocorticism): urine osmolality after H_2O deprivation < 600 mosmol/kg; 20%–50% increase in urine osmolality after ADH administration
3. Central diabetes insipidus cases: urine osmolality after H_2O deprivation < 300 mosmol/kg; > 100% increase in urine osmolality after ADH administration

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Chapter 9

MONOVALENT ELECTROLYTES AND OSMOLALITY

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Table 9.1. Abbreviations and symbols in Chapter 9

»	symbol in tables for relatively common disease or condition
[x]	concentration of x; x = analyte
ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
ADH	antidiuretic hormone
ANP	atrial natriuretic peptide
ATPase	adenosine triphosphatase
CK	creatine kinase
CRH	corticotropin releasing hormone
ECF	extracellular fluid
fCa^{2+}	free ionized calcium
fMg^{2+}	free ionized magnesium
GFR	glomerular filtration rate
GI	gastrointestinal
HCO_3^-	bicarbonate
ICF	intracellular fluid
IV	intravenous
mA^-	measured anion charge
mC^+	measured cation charge
M_r	relative molecular weight
$Na^+:K^+$	sodium to potassium
NADH	reduced nicotinamide adenine dinucleotide
Osm_c	calculated osmolality
Osm_m	measured osmolality
P_{CO_2}	partial pressure of carbon dioxide
PO_4	phosphate, all forms
RAS	renin-angiotensin system
SI	Système International d'Unités
SIADH	syndrome of inappropriate ADH secretion
SID	strong ion difference
SO_4	sulfate, all forms
tA^-	total anion charge
$tbCl^-$	total body chloride content
tbH_2O	total body H_2O content
tbK^+	total body potassium content
$tbNa^+$	total body sodium content
tC^+	total cation charge
tCO_2	total carbon dioxide content
uA^-	unmeasured anion charge
uA	unmeasured anion
uC^+	unmeasured cation charge
uC	unmeasured cation
UN	urea nitrogen
USG_{ref}	refractometric urine specific gravity
WRI	within reference interval

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D. Electrolytes and H_2O are excreted or lost from the ECF via kidneys, alimentary tract, skin, and airways. ECF tends to become hypotonic with loss of hypertonic fluids or hypertonic with loss of hypotonic fluids. Drinking H_2O after the loss of ECF will dilute the remaining ECF.

E. HCO_3^- concentrations are altered by changes in the concentrations of other electrolytes and by changes in acid-base balance.

III. Abnormal electrolyte concentrations in plasma or serum 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 kidneys, alimentary tract, skin, or airways (HCO_3^- indirectly)

SODIUM (Na^+) CONCENTRATION

I. Physiologic processes
  A. Serum [Na^+] is nearly equivalent to ECF [Na^+], which is dependent on the ratio of $tbNa^+$ to tbH_2O (Eqs. 9.1.a-9.1.c). Therefore, serum [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).

$$\text{Normonatremia} \Rightarrow \text{normal}\frac{tbNa^+}{tbH_2O} \Rightarrow \frac{\text{normal}}{\text{normal}} \textit{ or } \frac{\uparrow}{\uparrow} \textit{ or } \frac{\downarrow}{\downarrow} \qquad \textbf{(9.1.a.)}$$

$$\text{Hypernatremia} \Rightarrow \uparrow \frac{tbNa^+}{tbH_2O} \Rightarrow \frac{\uparrow}{\text{normal}} \textit{ or } \frac{\text{normal}}{\downarrow} \textit{ or } \frac{\downarrow}{\downarrow\downarrow} \qquad \textbf{(9.1.b.)}$$

$$\text{Hyponatremia} \Rightarrow \downarrow \frac{tbNa^+}{tbH_2O} \Rightarrow \frac{\downarrow}{\text{normal}} \textit{ or } \frac{\text{normal}}{\uparrow} \textit{ or } \frac{\downarrow\downarrow}{\downarrow} \qquad \textbf{(9.1.c.)}$$

  B. Movement of Na^+ frequently is associated with movement of H_2O in response to Na^+-induced changes in osmotic pressure; Na^+ retention tends to cause H_2O retention (edema, ascites) and Na^+ wasting tends to cause loss of H_2O (hypovolemia, dehydration). However, H_2O does not follow Na^+ across tubular cell membranes of the distal nephron in the absence of ADH or across membranes of the ascending limb of the loop of Henle.
  C. Serum [Na^+] is controlled through two major mechanisms: regulation of blood volume and regulation of plasma osmolality.
    1. Regulation of blood volume
      a. If hypovolemia is sensed by the kidneys (decreased effective blood volume), the RAS is activated, which promotes formation of angiotensin II in lungs and aldosterone in adrenal cortices. Angiotensin II stimulates the proximal tubular resorption of Na^+, Cl^-, and H_2O. Aldosterone stimulates the active resorption of Na^+ in collecting tubules 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 be one of the proteins).

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b. If hypovolemia is detected by carotid sinus baroreceptors, ADH secretion occurs.
c. If hypervolemia is sensed by atrial baroreceptors, Na^+ resorption in the distal nephron is reduced through the action of ANP, which reduces the number of open Na^+ channels via the guanylate cyclase pathway.

2. Regulation of plasma osmolality
   a. If hyperosmolality is sensed by hypothalamic osmoreceptors, thirst centers are stimulated to promote H_2O intake 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 through the Na^+-K^+-$2Cl^-$ carrier.
   b. Conversely, hypoosmolality leads to decreased H_2O intake and increased urinary H_2O excretion.

D. Na^+ and Cl^- resorption in the distal nephron also involves an aldosterone-independent Na^+-Cl^- cotransporter, a process in which increased Na^+ delivery to the distal nephron results in increased resorption. Thiazide diuretics block this cotransporter by binding to the Cl^- receptor.

E. 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 H_2O deficit (without Na^+ deficit) include decreased H_2O intake, free-H_2O loss (central diabetes insipidus, nephrogenic diabetes insipidus), and insensible respiratory losses.
      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
      a. Hypernatremic, hyperosmolar or hypertonic dehydration is caused by net hypoosmolar or hypotonic fluid loss: i.e., H_2O loss > Na^+ loss.
      b. Normonatremic, isoosmolar, or isotonic dehydration is caused by net isoosmolar or isotonic fluid loss: i.e., H_2O loss ≈ Na^+ loss.
      c. Hyponatremic, hypoosmolar, or hypotonic dehydration is caused by net hyperosmolar or hypertonic fluid loss: i.e., H_2O loss < Na^+ loss.

II. Analytical concepts

A. Terms and units
   1. Assays measure the electrical potential of Na^+ (potentiometry) or $[Na^+]$ (flame photometry) in plasma or serum; then the electrical potential is converted to concentration units. The Na^+ is a free ion in the plasma or serum H_2O.
   2. Unit conversion: mEq/L × 1 = mmol/L; mg/dL ÷ 2.3 = mmol/L (SI unit, nearest 1 mmol/L)[1]

B. Sample for $[Na^+]$ quantitation
   1. Serum is preferred; $[Na^+]$ is stable for months if the sample does not dehydrate.
   2. Na_2EDTA plasma should not be used because the anticoagulant's Na^+ will be in the plasma. The amount of Na^+ in Na-heparin is too small to cause clinically relevant changes in heparinized plasma $[Na^+]$.

C. Assays
   1. Ion-selective electrode assays are the most common.
   2. Flame photometers for measuring $[Na^+]$ used to be the gold standard but the instruments are essentially obsolete.

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Table 9.2. Diseases and conditions that cause hypernatremia

- H_2O-deficit group (decreased total body H_2O)
  - Inadequate H_2O intake
    - [a]H_2O deprivation
    - Defective thirst response (hypothalamic defect)
  - Pure H_2O loss (without adequate H_2O replacement)
    - [a]Insensible loss: panting, hyperventilation, or fever
    - Diabetes insipidus (central or nephrogenic)
  - H_2O loss > Na^+ loss
    - Renal: osmotic diuresis
    - Alimentary: osmotic diarrhea, osmotic sequestration, or phosphate enemas
- Na^+-excess group (increased total body Na^+)
  - Excess Na^+ with concurrent restricted H_2O intake
    - Salt poisoning
    - Administration of hypertonic saline or sodium bicarbonate
  - Decreased renal excretion of sodium
    - Hyperaldosteronism
- Other or unknown mechanism
  - Severe exercise in greyhounds

Note: Sample evaporation or sublimation will cause spurious hypernatremia.

III. Hypernatremia

A. Hypernatremia occurs when the ratio of $tbNa^+$ to tbH_2O is increased (Eq. 9.1.b). It also may occur 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): Dehydration may cause hypernatremia directly via hemoconcentration (pure H_2O loss) and indirectly by activating the RAS, which stimulates renal Na^+ retention via actions of aldosterone and angiotensin II. In most nephron segments, Na^+ resorption promotes H_2O resorption. But since the distal nephron is 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: Restricted access to H_2O may occur because of accidents (H_2O bowl turned over on a hot day), weather (frozen H_2O 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: Hypothalamic disease may damage the osmoreceptor that triggers a thirst response or the thirst center itself may be damaged.

b. Pure 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)

(a) In the diabetes insipidus disorders, diminished ADH activity or response in the collecting ducts may result in pure H_2O loss (i.e., urine with very low concentrations of Na^+ and other solutes; USG_{ref} approaches 1.000).

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(b) Animals with diabetes insipidus that have unrestricted access to H_2O may drink sufficiently to prevent the hypernatremia.

c. H_2O loss > Na^+ loss

(1) Osmotic diuretic agents (e.g., glucose, mannitol) in renal tubular fluid inhibit passive H_2O resorption.

(2) In the alimentary system,

(a) Accumulation of osmotic agents (as occurs in some diarrheas) will inhibit H_2O absorption.

(b) A phosphate enema will pull H_2O from ECF spaces to the colon; there may be a concurrent colonic absorption of Na^+ that augments the hypernatremia.[2]

(c) In ruminal acidosis (grain overload), accumulation of solutes (including lactic acid) in the rumen causes the osmotic movement of H_2O into the rumen to produce hypernatremia.[3]

(3) The H_2O that moves during osmosis contains a small amount of Na^+ because of solute drag, but the net result is greater loss of H_2O than Na^+ and thus hypernatremia occurs.

(4) Unrestricted access to H_2O may prevent this hypernatremia from occurring. Also, other processes in such cases (e.g., ketosis) may increase renal Na^+ loss and counteract hypernatremic tendencies.

2. Na^+-excess group (increased tbNa^+)

a. Excess Na^+ 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 tbCl^-) and thus hypernatremia (and hyperchloremia). Extreme hypernatremia and hyperchloremia occurred in a dog that ingested a salt-flour mixture that was used as modeling clay.[4]

(2) Administration of hypertonic saline or sodium bicarbonate can lead to increased tbNa^+ content and thus hypernatremia (and hyperchloremia or increased HCO_3^-).

b. Decreased renal excretion of sodium (hyperaldosteronism) (rare)

(1) Excessive aldosterone promotes excessive renal Na^+ (and Cl^-) retention.

(2) Hypernatremia (and hyperchloremia) may occur if there is H_2O restriction or defective ADH activity.

3. Other or unknown mechanisms

a. Severe exercise (racing greyhounds):[5-7] During and immediately after a race, [Na^+] in plasma may be 10–20 mmol/L above prerace concentrations. There is a concurrent hypovolemia and lactic acidosis, thus the hypernatremia may be due to shifting of H_2O from ECF to ICF. The accumulation of lactate in muscle fibers may create the osmotic gradient.[8]

b. Sample dehydration: Exposure of serum or plasma to air may allow evaporation that results in hypernatremia. This is especially true of air-conditioning systems that blow cool dry air over the sample processing or analysis areas. Sublimation of H_2O from frozen samples may result in hypernatremia.

IV. Normonatremia in dehydrated or edematous animals

A. Normonatremia does not necessarily indicate that Na^+ balance is normal (Eq. 9.1.a). Recognizing the possibility of altered Na^+ regulation in normonatremic animals is impor-

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Table 9.3. Diseases and conditions that cause normonatremia in dehydrated or edematous animals

Net loss of isotonic fluids causing dehydration
ªAlimentary losses: vomiting, diarrhea, sequestration
ªRenal losses: renal disease, osmotic diuresis, diuretics
Skin loss: sweating in horses
Net retention of isotonic fluids causing edema or transudate
ªCongestive heart failure
ªHepatic cirrhosis
ªNephrotic syndrome

tant in the management and treatment of these cases (i.e., do you need to administer Na^+ or restrict Na^+ intake?).

B. Disorders and pathogeneses (Table 9.3)
   1. Net loss of isotonic fluids causing 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 causes impaired resorption of H_2O in tubules. A high tubular flow rate also contributes to Na^+ loss.
         (3) Most diuretic agents (furosemide, thiazides) cause a loss of Na^+ and H_2O, both through interfering with Cl^- resorption.
      c. Cutaneous loss in horses: $[Na^+]$ in sweat is about the same as $[Na^+]$ in serum or plasma. Thus, profuse sweating without increased H_2O intake could cause normonatremic dehydration.
   2. Net retention of isotonic fluids causing 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 (due to valvular disease or cardiomyopathies)
         (1) "Forward" hypothesis: Cardiac disease causes decreased cardiac output, which is sensed by baroreceptors as decreased effective blood volume, and thus there is stimulation of the sympathetic nervous system and the RAS. If these responses do not re-establish the effective blood volume, continued activation of RAS promotes renal resorption of Na^+ and Cl^-, which increases plasma osmolality. Hyperosmolality stimulates release of ADH and the thirst center, which may cause retention of H_2O and increased blood volume. If the hypervolemia increases venous hydrostatic pressure sufficiently, it promotes movement of H_2O to extravascular spaces and thus formation of edema (pulmonary or dependent) or accumulation of H_2O in pleural or peritoneal cavities (transudation).
         (2) Retention of Na^+ and H_2O is a compensatory process that helps maintain an effective blood volume. If not achieved, the hypovolemic stimulus may promote thirst and thus increased H_2O intake. Mammals with a cardiac disease that causes decreased cardiac output will have increased $tbNa^+$ and tbH_2O even without clinical edema.

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b. Hepatic cirrhosis with abdominal transudation
   (1) Three theories (underfilling, overflow, and peripheral arterial vasodilation) attempt to explain the peritoneal transudation that occurs with hepatic cirrhosis.[9,10]
      (a) In the underfilling theory, the initiating event is the loss of plasma H_2O caused by increased hydrostatic pressure in hepatic sinusoids (caused by fibrosis or venous congestion) and loss of protein-rich plasma into the space of Disse (sinusoids are highly permeable to albumin). This causes splanchnic pooling of blood and loss of H_2O across the hepatic capsule to the peritoneal cavity and thus "underfilling" of vascular spaces. Movement of fluid from vessels results in a decreased effective blood volume, which activates the RAS, stimulates release of aldosterone, promotes retention of Na^+, and subsequently H_2O. The expanded blood volume accentuates vascular hydrostatic pressures and thus promotes more loss of plasma H_2O and more attempts to compensate. At the time of clinical transudation, the animal will have increased $tbNa^+$ and tbH_2O, increased concentrations of renin, norepinephrine, and ADH, and reduced renal excretion of Na^+.
      (b) In the overflow theory, the initiating event is renal retention of Na^+ and H_2O due to poorly understood factors.
      (c) In the peripheral arterial vasodilation theory, peripheral vasodilation creates a decreased effective blood volume, which activates the RAS. This leads to increased hydrostatic pressures in hepatic sinusoids, which leads to transudation.
   (2) Hypoalbuminemia and hypoproteinemia play secondary roles in the ascites formation.
      (a) In health, plasma proteins create colloidal osmotic (oncotic) pressure (about 80% from albumin and 20% from globulins) that helps retain H_2O in vessels that are not permeable to proteins. When hypoproteinemia is present with hepatic cirrhosis, the increases in vascular hydrostatic pressure have more effect 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 they do not have generalized transudation because of compensatory processes (increased globulins, altered capillary hydrostatic pressure, altered renal blood flow).[11,12] Also, hepatic sinusoids are freely permeable to albumin and H_2O and thus movement of plasma H_2O out of the sinusoids occurs in either the presence or absence of hypoalbuminemia.

c. Nephrotic syndrome (protein-losing nephropathy that leads to abdominal transudation)[8,13]
   (1) Pathogenesis of Na^+ and H_2O retention is not established but involves several processes: increased activity of the RAS, decreased renal responsiveness to ANP, decreased 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.

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Table 9.4. Diseases 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)
  - ”Alimentary loss: vomiting, diarrhea, sequestration, canine whipworm infection, excess salivation
  - ”Renal loss: hypoadrenocorticism, prolonged diuresis, ketonuria, Na^+-wasting nephropathies
  - Cutaneous loss: sweating in horses
  - Third-space loss: acute hemorrhage, acute exudation, repeated drainage of chylous effusion
- H_2O-excess groups (with or without edema)
  - Edematous disorders
    - ”Congestive heart failure
    - ”Hepatic cirrhosis
    - ”Nephrotic syndrome
  - Expanded ECF volume (but without edema)
    - SIADH
    - Excess administration of Na^+-poor fluids
- 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

Note: Pseudohyponatremia may be caused by displacement of serum or plasma H_2O (see text).

V. Hyponatremia

A. Hyponatremia typically occurs when the ratio of $tbNa^+$ to tbH_2O is decreased (Eq. 9.1.c); it may also occur with 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. (In the following disorders, $[Na^+]$ in lost fluid is usually not greater than plasma $[Na^+]$, but loss of Na^+-containing fluid followed by drinking of H_2O may result in hyponatremia.)

a. Alimentary loss of Na^+-containing H_2O may occur with vomiting, diarrhea, sequestration, and excess salivation, and in canine whipworm infections. 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 results in a dilution of remaining plasma Na^+.

b. Renal loss

(1) Hypoadrenocorticism:[8] Adrenal insufficiency causes a decreased aldosterone concentration, which causes less resorption of Na^+ and subsequently Cl^- by the renal principal cells. 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 lead to dilution of ECF Na^+ and thus hyponatremia (and hypochloremia). With concurrent hypochloremia, the

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resulting hypoosmolality tends to reduce ADH secretion. Hypocortisolemia of adrenal insufficiency also contributes to the hyponatremia but the mechanism is not established. Hypocortisolemia promotes the hypothalamic release of ADH (multiple theories), which results in a defective excretion of H_2O (retain H_2O in the presence of hypoosmolality) and a dilutional hyponatremia.[14] In people with hypoaldosteronism and lack of hypocortisolemia, normonatremia is maintained by enhanced tubular resorption of Na^+ caused by increased angiotensin II, decreased ANP, and enhanced passive resorption.[8]

(2) Prolonged diuresis
   (a) Prolonged osmotic (crystalloid solutions)[15] or other forms of diuresis (furosemide)[16] tend to cause depletion of Na^+ and H_2O, hypovolemia, and stimulation of ADH release and thirst centers. H_2O drinking 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 distal nephrons. Urinary K^+ loss is increased because of an increased flow rate in distal nephrons and hypovolemia-induced release of aldosterone, which opens K^+ channels. The hypovolemia also stimulates ADH release, which promotes free-H_2O resorption and thus dilution of the remaining Na^+, Cl^-, and K^+.

(3) Ketonuria
   (a) During ketonuria, there is increased excretion of ketone bodies (acetoacetate and β-hydroxybutyrate). The presence of these nonabsorbable anions in the tubular lumen obligates the excretion of cations and thus increased renal excretion of Na^+.
   (b) Concurrent osmotic diuresis (from glucosuria) may compound the Na^+ loss associated with ketonuria in patients with diabetes mellitus.

(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.

c. Cutaneous loss due to sweating
   (1) Among the domestic mammals, only the horse sweats sufficiently to cause electrolyte and H_2O imbalances.
   (2) Equine sweat is a Na^+-, K^+-, and Cl^--rich fluid (concentrations are greater than plasma concentrations but evaporation may contribute to the increases).[17,18] Drinking of H_2O or the ADH-stimulated retention of H_2O after sweating may lead to dilutional hyponatremia.

d. Third-space loss (typically 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.[19]
      (b) This type of hyponatremia would probably result from repeated removal of other third-space fluid but such removal is not as common as it is for chylous effusions.
   (2) Acute internal hemorrhage or acute exudation

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Na^+ in the total plasma sample is less, and thus the measured [Na^+] is decreased.

(2) Indirect potentiometry: Ion-selective electrodes measure the Na^+ potential in substantially diluted serum or plasma samples. If excess solids are present in the plasma or serum, the amount of plasma H_2O in the diluted sample will be less than usual, so the measured electrolyte activity will be lower than if the same volume of sample with normal solids was diluted and tested. A back-calculation is then used to correct for the dilution, but the dilution factor is based on the total volume of the plasma or serum used in the dilution, not on just its aqueous phase. Therefore, the back-calculation results in a falsely low value.[24]

b. Laboratories may measure serum or plasma [Na^+] by direct potentiometry using ion-selective electrodes. Such assays do not require sample dilution so excess solids in the plasma do not affect them. The electrodes detect the Na^+ activity in the aqueous phase only.

POTASSIUM (K^+) CONCENTRATION

I. Physiologic processes

A. Serum [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, 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.

C. Acidoses and alkaloses alter serum [K^+](Fig. 9.2).

1. An inorganic or mineral acidosis (renal failure, some diarrheas) may cause hyperkalemia because of the shifting of K^+ out of cells.

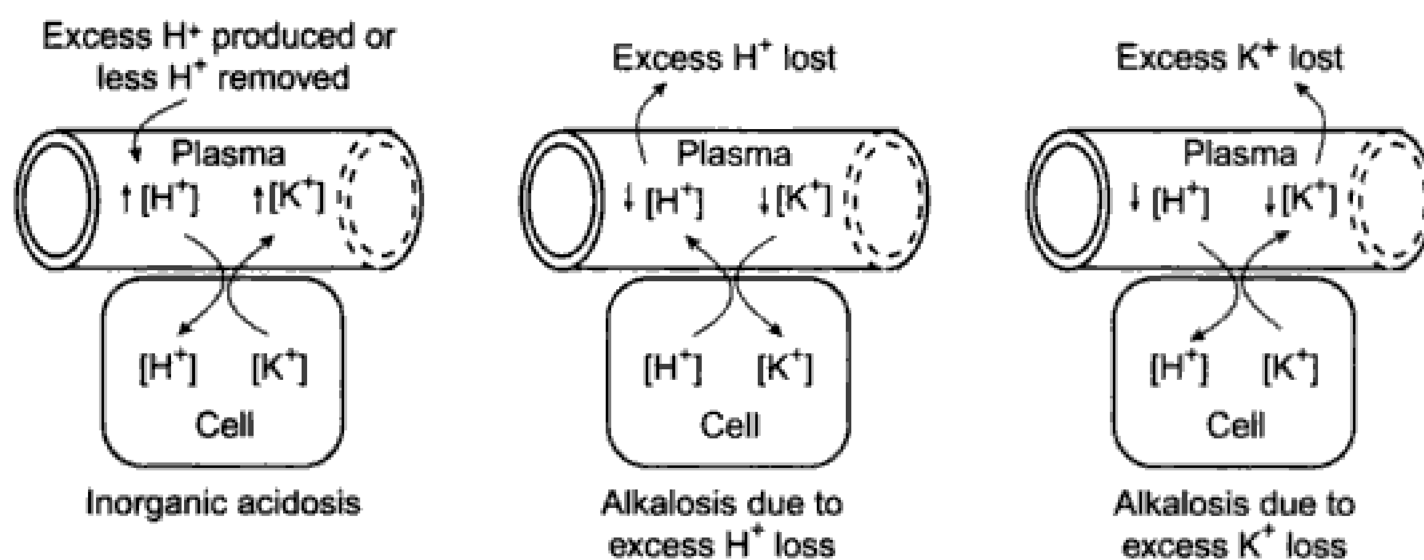

Fig. 9.2. H^+ and K^+ shifts in acid-base disorders.

In an inorganic acidosis, there is an accumulation of H^+ in ECF. As H^+ shifts into cells to equilibrate concentrations, K^+ shifts out of cells and enters plasma (hyperkalemia occurs) to maintain electrical neutrality.[60] In an organic acidosis, a hyperkalemia is not expected because additional factors influence plasma [K^+]. In an alkalosis, there is a depletion of H^+ in ECF. As H^+ shifts out of cells to equilibrate concentrations, K^+ leaves the plasma and enters cells (hypokalemia occurs). Conversely, if K^+ depletion causes hypokalemia, K^+ shifts out of the cells and H^+ enters and thus lowers blood [H^+] (alkalemia).

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2. An organic acidosis (e.g., lactic acidosis and ketoacidosis) typically does not cause hyperkalemia but the reasons may vary.
   a. If an anion (such as lactate or ketone body) enters the cells when H^+ enters the cell, electroneutrality is maintained and thus K^+ does not need to leave the cell.
   b. Acidemia may promote the loss of K^+ from cells but the K^+ is excreted in urine with the organic anions (lactate, acetoacetate) that are also excreted during lactic and ketoacidosis, respectively.
3. Treatment of an acidotic state may result in hypokalemia that reflects tbK^+ depletion in the animal.
4. A metabolic alkalosis may result in mild hypokalemia.
5. Respiratory acidoses and alkaloses are not associated with altered serum $[K^+]$,[8] maybe because other regulatory systems are still functional.

D. If the acid-base status is normal, then serum or plasma $[K^+]$ tends to reflect tbK^+. That is, hyperkalemia tends to occur with increased tbK^+, and hypokalemia tends to occur with decreased tbK^+. However, a K^+-depleted state may occur before hypokalemia develops.[25]

E. Regulation of plasma $[K^+]$ occurs through two major processes: (1) distribution between ECF and ICF, and (2) renal excretion.
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^+; 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, $[K^+]$ in tubular fluid entering the distal nephron is near zero.
   b. K^+ secretion occurs primarily in the principal cells of the collecting tubules (Fig. 9.3). Aldosterone promotes this process by stimulating a Na^+-K^+-ATPase in the basolateral membrane and opening luminal K^+ channels. Hyperkalemia and angiotensin II are the major stimulants of aldosterone secretion. ACTH and hyponatremia also stimulate aldosterone release.[8]
   c. High flow rate of tubular fluid also promotes K^+ excretion 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^+ excretion.
   d. Resorption of Na^+ in the distal nephron (if not accompanied by Cl^-, e.g., hypochloremic states) establishes an electrochemical gradient (tubular fluid more negative than cell) that promotes the secretion of K^+.
3. K^+ is conserved by Type A intercalated cells of the distal nephron through H^+-K^+-ATPase pump activity when there is K^+ depletion (Fig. 9.4).
4. ADH promotes K^+ secretion that counterbalances the reduced K^+ secretion that occurs with decreased urinary flow. The enhanced secretion prevents the hyperkalemia that might result from dehydration-induced oliguria.
5. 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. Terms and units
1. Assays measure the electrical potential of K^+ (potentiometry) or $[K^+]$ (flame photom-

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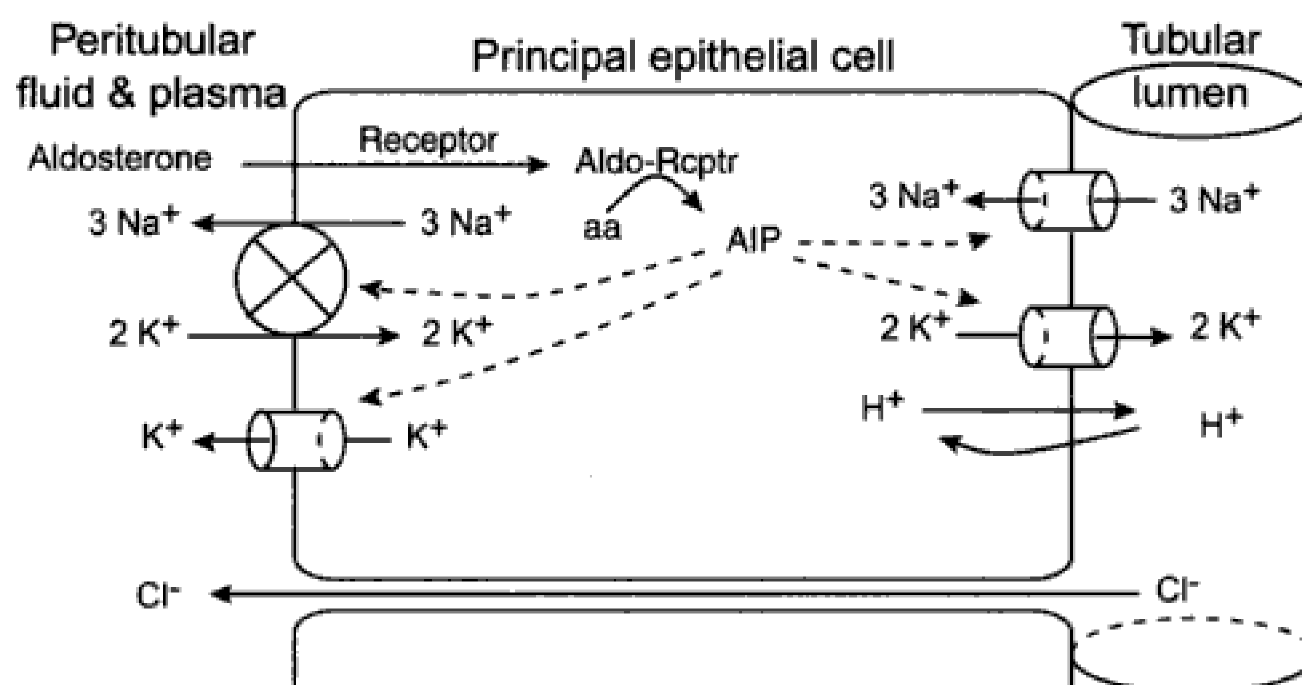

Fig 9.3. Actions of aldosterone on principal epithelial cells in cortical collecting tubules. (⊗ = ATPase pump)
Aldosterone enters the principal cells and binds to receptor proteins. The aldosterone-receptor complex (Aldo-Rcptr) stimulates the synthesis of aldosterone-induced proteins (AIP) that may include components of 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 Na^+ channel.
2. K^+ is pumped into the cell from the peritubular fluid and typically exits to the tubular fluid through an opened K^+ channel. When hypokalemia is present, K^+ may return to the peritubular fluid via an opened basolateral membrane channel (recycled).
3. Typically, the Na^+-K^+-ATPase pump results in a net negative charge in the tubular fluid (3 Na^+ resorbed and 2 K^+ secreted). The negative charge promotes the resorption of Cl^- through a paracellular route. When there is less Cl^- available (as occurs with hypochloremia), the negative charge promotes the retention of H^+ in the tubular fluid (the H^+ that passively leaves and re-enters 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^+. In the presence of hypochloremia, there is increased excretion of H^+, which can promote aciduria or a metabolic alkalosis.

etry) in plasma, serum, or whole blood; then electrical potential is converted to concentration units. The K^+ is a free ion in the plasma or serum H_2O.

2. Unit conversion: mEq/L × 1 = mmol/L; mg/dL ÷ 3.9 = mmol/L (SI unit, nearest 0.1 mmol/L)[1]

B. Sample for [K^+] quantitation
   1. Serum is preferred; [K^+] is stable for months if the sample does not dehydrate.
   2. K_3EDTA should not be used as an anticoagulant for testing plasma [K^+] because the anticoagulant's potassium will be added to the plasma and be measured.

C. Assays
   1. Ion-selective electrode assays are the most common.
   2. Flame photometers for measuring [K^+] used to be the gold standard but the instruments are essentially obsolete.

III. Hyperkalemia

A. Hyperkalemia typically occurs when there is decreased renal excretion of K^+ or a shift of K^+ from the ICF to ECF; hyperkalemia may also occur with increased intake of K^+ or

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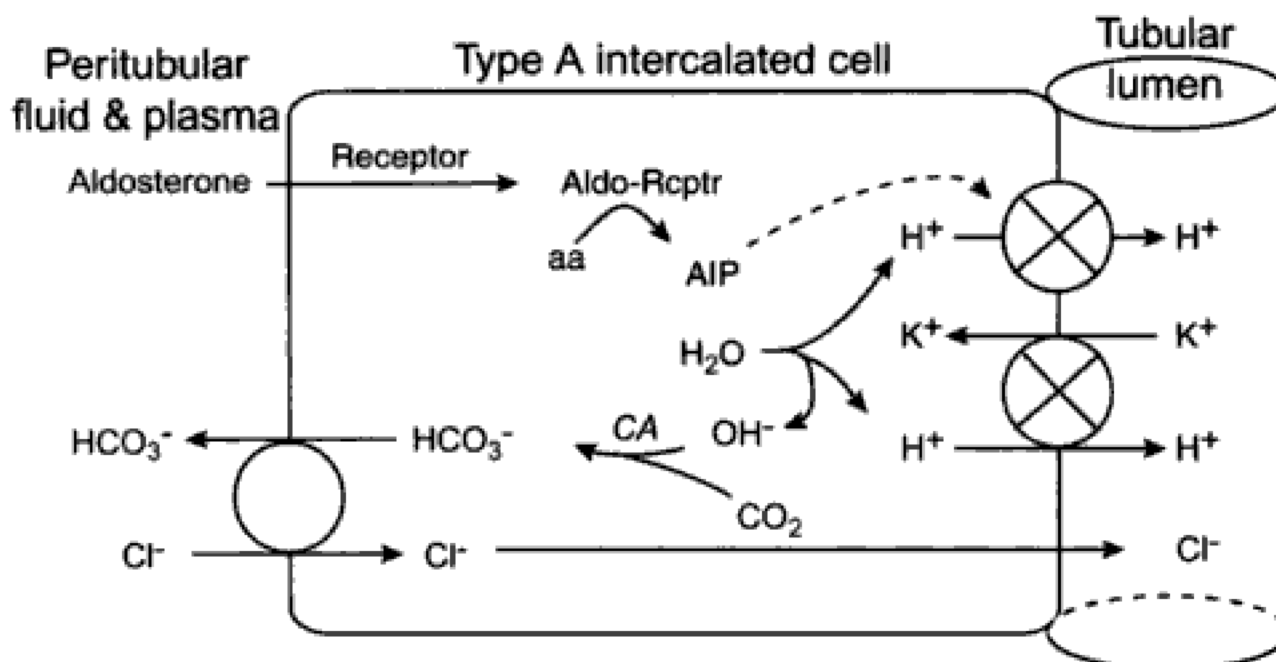

Fig 9.4. Secretion of H^+ by Type A intercalated cells of distal nephron. (⊗ = ATPase pump, ◯ = cotransporter or counter transporter [antiporter])

Aldosterone enters the Type A intercalated cells and binds to receptor proteins. The aldosterone-receptor complex (Aldo-Rcptr) stimulates the synthesis of aldosterone-induced proteins (AIP) that may include components of 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 (*CA*) to form HCO_3^-, which is exchanged for Cl^- in the peritubular fluid.
3. Cl^- that enters the cell in the Cl^--HCO_3^- exchanger is secreted into the tubular lumen to maintain electrical neutrality (balance the H^+ secretion).

The net result if stimulated by acidemia or aldosterone is increased H^+ and Cl^- excretion and increased HCO_3^- in plasma.

In an independent process but in the same cells, a H^+-K^+-ATPase promotes the resorption of K^+ and secretion of H^+; this pump is more active when hypokalemia is present. Therefore, hypokalemia promotes increased H^+ excretion, HCO_3^- production, and thus alkalosis.

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
      a. Metabolic inorganic acidoses: shifting of K^+ out of cells when H^+ moves in (see I.C in this section)
      b. Massive tissue necrosis: release of K^+-rich ICF from dead cells; seen with tumor necrosis and sometimes just prior to death
      c. Rhabdomyolysis or other muscle damage (release of K^+ from muscle fibers)
         (1) Acquired disorders: selenium deficiency,[21] malignant hyperthermia,[26] seizures
         (2) Congenital disorders: stress or anesthetic-induced in dystrophin-deficient cats,[27] hyperkalemic periodic paralysis in quarter horses,[28] and possibly hyperkalemic periodic paralysis in a dog (serum $[K^+]$ was never documented above 5 mmol/L; serum $[K^+]$ did increase from 3.9 mmol/L to 4.9 mmol/L after exercise)[29]
      d. Massive intravascular hemolysis in animals with K^+-rich erythrocytes. English Springer Spaniels with phosphofructokinase deficiency have hyperkalemia concurrent with their hemolytic episodes that may involve K^+-rich young erythrocytes.[30]

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Table 9.5. Diseases and conditions that cause hyperkalemia

Diseases and conditions
Shifting of K^+ from ICF to ECF (no change in total body K^+)
[a]Metabolic acidoses due to accumulation of inorganic acids
Massive tissue necrosis
Rhabdomyolysis or other muscle damage
Acquired: strenuous exercise, seizures, selenium deficiency
Congenital: hyperkalemic periodic paralysis in quarter horses and perhaps dogs
Massive intravascular hemolysis in some animals (see text)
Increased total body K^+
Decreased renal excretion of K^+
[a]Renal insufficiency or failure (primarily oliguric or anuric)
[a]Urinary tract obstruction or leakage into body
Hypoaldosteronism
[a]Hypoadrenocorticism (Addison's disease)
ACE inhibitors
Trimethoprim-induced K^+ retention
Increased intake
Administration of K^+-rich fluid
Other or unknown mechanism
Repeated drainage of chylous thoracic effusions
Peritoneal effusion

Note: Also consider pseudohyperkalemia (see text).

e. Pseudohyperkalemia
  (1) *In vitro* hemolysis or leakage of K^+ resulting from delayed removal of serum or plasma from erythrocytes
    (a) If the $[K^+]$ in erythrocytes is greater than the $[K^+]$ in plasma, then loss of K^+ from erythrocytes may cause pseudohyperkalemia. The ratio of erythrocyte $[K^+]$ to plasma $[K^+]$ varies between species and between breeds of dogs.
    (b) This form of pseudohyperkalemia may occur in horses,[31] some dogs (e.g., Japanese Akita[32] and Japanese Shiba), and cattle.
    (c) Hemolysis is not expected to cause hyperkalemia in cats or other breeds of dogs.
  (2) Thrombocytosis[33]
    (a) K^+ is released from platelets in the clotted blood sample; the released K^+ is part of the $[K^+]$ used to establish serum reference intervals. Without a thrombocytosis, serum $[K^+]$ is about 0.3–0.5 mmol/L higher than plasma $[K^+]$ from the same blood sample.
    (b) When there is a marked thrombocytosis (> 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.
  (3) Leakage of K^+ from leukemic cells[34]

2. Increased total body K^+
  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)

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not established but may be related to concurrent hyponatremia. In the presence of hyponatremia and hypovolemia, much of the filtrate Na^+ is resorbed in the proximal tubules and ascending loop of Henle. If Na^+ is not delivered to the distal nephron, less Na^+ is resorbed distally and thus less K^+ excretion occurs.

4. Peritoneal effusions: Four cats with peritoneal effusions had hyperkalemias and either mild or marked hyponatremias.[38] The effusions were due to abdominal carcinomatosis (one cat) and feline infectious peritonitis (two and possibly three cats; firm diagnosis not made in the last cat). The hyperkalemia may be due to impaired renal excretion of K^+ because of decreased distal tubular flow rate and concurrent hyponatremia. The hyponatremia was considered a dilutional hyponatremia that resulted from H_2O retention caused by stimulation of ADH release and thirst centers by a decreased effective blood volume.

IV. Normokalemia in acidotic or alkalotic animals
   A. Normokalemia in an acidotic animal
      1. Because inorganic acidemia is expected to increase serum [K^+], normokalemia in the presence of inorganic acidemia suggests the animal has a decreased tbK^+. Therapeutic correction of the inorganic 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, animals with an organic metabolic acidosis may not have a hyperkalemia because the K^+-shift into cells is accompanied by an organic anion shift or there is increased renal excretion of K^+ because of the organic anion excretion.
   B. Normokalemia in an alkalotic animal
      1. Because alkalemia is expected to decrease serum [K^+], normokalemia in the presence of alkalemia suggests the animal has an increased tbK^+. Therapeutic correction of the alkalemia may raise the serum [K^+] into the hyperkalemic range.
      2. This combination of findings is not expected because disorders that cause metabolic alkalosis typically do not concurrently cause increased tbK^+.

V. Hypokalemia
   A. Hypokalemia typically occurs when there is a shift of K^+ from the 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 serum [K^+] tends to reflect tbK^+.
   B. Disorders and pathogeneses (Table 9.6)
      1. Shifting of K^+ from ECF to ICF
         a. Metabolic alkalosis: shifting of K^+ into cells when H^+ moves out (see I.C in this section).
         b. Increased insulin activity: Insulin promotes the cellular uptake of K^+, probably through the activation of a Na^+-K^+-ATPase. Administration of exogenous insulin or a sudden release of endogenous insulin after intravenous glucose administration may cause hypokalemia. (Also see Ketonuria a few paragraphs below.)
      2. Decreased total body K^+
         a. Decreased K^+ intake
            (1) Because most diets are K^+-rich, anorexia (or other reasons for not eating) can contribute to decreased tbK^+.
            (2) The severity of hypokalemia will be enhanced if other processes are leading to K^+ loss or shifting to ICF.

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Table 9.6. Diseases and conditions that cause hypokalemia

- Shifting of K^+ from ECF to ICF (no change in total body K^+)
  - Metabolic alkalosis
  - Increased insulin activity (rapid increase)
- Decreased total body K^+
  - [a]Decreased K^+ intake: anorexia or other reasons for not eating
  - Increased excretion of K^+
    - Increased renal loss
      - [a]Increased fluid flow in distal nephron: osmotic, Na^+-losing nephropathies, or therapeutic diuresis (including loop and thiazide diuretics)
      - [a]Ketonuria
      - [a]Vomiting or sequestration of H^+ and Cl^- causing hypochloremic metabolic alkalosis
      - Hyperaldosteronism, primary
    - [a]Increased alimentary loss: vomiting, diarrhea, sequestration
    - Increased cutaneous loss: sweating in horses
  - Other or unknown mechanisms
    - [a]Renal failure in cats
    - Hypokalemic myopathy of Burmese kittens

b. Increased excretion of K^+
   (1) Increased renal loss
      (a) Increased tubular flow
         (i) Increased flow of fluid through the collecting tubules allows for increased excretion 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, possibly hypercalcemic nephropathy), and diuretic use (loop and thiazide).
      (b) Ketonuria
         (i) Acetoacetate and β-hydroxybutyrate (ketone bodies) are anions that are not resorbed in the tubules. Their negative charges add to the electrochemical gradient that promotes K^+ secretion, especially when there is concurrent stimulation of Na^+ resorption.
         (ii) Animals with ketoacidotic diabetes mellitus tend to be hypokalemic; however, the net K^+ balance is the result of multiple concurrent processes.
      (c) Vomiting or sequestration of H^+ and Cl^- causing hypochloremic metabolic alkalosis (e.g., displaced abomasum or other upper GI obstruction)
         (i) During the development of this metabolic alkalosis, plasma $[HCO_3^-]$ may increase sufficiently 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) K^+ (and concurrently H^+) secretion is promoted through the actions of aldosterone if the animal is hypovolemic.

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(iii) Some K^+ in gastric secretions may be lost via vomiting.

(d) Hyperaldosteronism, primary[39]

(i) Dogs with hyperaldosteronism due to adrenal neoplasms may have hypokalemia.[40] A similar pathologic state has been reported in cats.[41,42]

(ii) The increased aldosterone concentrations would promote renal K^+ secretion by stimulating Na^+-K^+-ATPase and opening K^+ channels in the collecting tubules (Fig. 9.3).

(2) Increased alimentary loss of K^+ via vomiting, diarrhea, sequestration, or excess salivation

(a) Vomiting may cause loss of K^+-rich fluid and thus lead to hypokalemia, but renal excretion may add to a decreased tbK^+.

(b) With diarrheas, massive electrolyte and H_2O losses may occur. 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 (choke, lacerated salivary gland, dysphagia, ptyalism) in horses and cattle can produce hypokalemia.[43] Initially, the concurrent loss of HCO_3^- may produce a mild acidosis. If the animals also become hyponatremic, hypochloremic, and hypovolemic, then renal compensations (conserve Na^+ and HCO_3^-, secrete 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.[17,18]

(b) If the horse is drinking, H_2O intake would promote dilution of the remaining K^+.

c. Other or unknown mechanisms

(1) Renal failure in cats

(a) Cats with progressive renal disease that results in chronic renal failure are prone to develop hypokalemia.

(b) The exact pathogenesis of the hypokalemia of chronic renal failure is unknown and may be due to multiple factors: increased renal excretion, increased colonic excretion, and decreased dietary intake.

(2) Hypokalemic myopathy of Burmese kittens[44-46]

(a) Affected Burmese kittens had hypokalemia and muscle weakness that was precipitated by stress or exercise; affected cats had high serum CK activities.

(b) The pathogenesis of the hypokalemia is not established but may be due to a sudden shift of K^+ from the ECF to ICF.

SODIUM:POTASSIUM (Na^+:K^+) RATIO

I. Because several physiologic processes involve both Na^+ and K^+, it's not surprising that concurrent changes in [Na^+] and [K^+] may occur in the same animal. Calculating a Na^+:K^+ ratio may enhance detection of electrolyte disorders.

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CHLORIDE (Cl^-) CONCENTRATION

I. Physiologic processes

A. Serum [Cl^-] is nearly equivalent to ECF [Cl^-], which is influenced by ECF concentrations of Na^+ and HCO_3^-. Therefore, complete interpretation of serum [Cl^-] requires knowledge of serum [Na^+] and at least a consideration of the animal's acid-base status.

B. Control of serum [Cl^-]

1. Renal resorption and excretion of Cl^-

a. About 75% of filtered Cl^- is resorbed in the proximal tubules down a concentration gradient created by Na^+ and H_2O resorption and through a formate-chloride exchanger. Angiotensin II stimulates the proximal tubular resorption of Na^+, Cl^-, and H_2O.

b. Cl^- is actively resorbed in the thick ascending limb of the loop of Henle via a Na^+-K^+-$2Cl^-$ transporter in the luminal membrane; the rate-limiting factor is Cl^- delivery to the loop. 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 distal nephron.

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 distal nephron. Thiazide diuretics block this cotransporter.

e. Type A intercalated cells of the distal nephron excrete Cl^- when H^+ is excreted; the process is stimulated by acidemia (Fig. 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 (illustration of processes in Type B cells not provided).

2. Alimentary tract functions pertaining to Cl^-

a. Gastric (or abomasal) mucosa secretes HCl as part of the digestive process. In the healthy animal, the secreted Cl^- is resorbed after it passes into the intestinal tract.

b. Secretion of Cl^- requires the generation of HCO_3^- (see Fig. 9.5).

II. Analytical concepts

A. Terms and units

1. Assays measure the electrical potential of Cl^- (potentiometry) or [Cl^-] (flame photometry) in plasma, serum, or whole blood; the Cl^- is a free ion in the plasma or serum H_2O.

2. Unit conversion: mEq/L × 1 = mmol/L; mg/dL ÷ 3.55 = mmol/L (SI unit, nearest 1 mmol/L)[1]

B. Sample for [Cl^-] quantitation

1. Serum is preferred; [Cl^-] is stable for months if the sample does not dehydrate.

2. Plasma may be used.

C. Principles of assays

1. Ion-selective electrode assays are the most common.

2. Other halides (e.g., bromide and iodide) will react with the electrode to give a falsely increased [Cl^-]. Bromide may be present when KBr is used as an anticonvulsant.

III. Hyperchloremia

A. Hyperchloremia typically occurs when there is hypernatremia or when there is a decreased [HCO_3^-] (as occurs with metabolic acidoses). Generally, changes in [Cl^-] are

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b. Renal loss of HCO_3^-
   (1) In proximal tubular acidosis, the reduced reclamation of HCO_3^- may allow more Cl^- to be resorbed with Na^+ and thus hyperchloremic metabolic acidosis develops.
   (2) In distal tubular acidosis, the impaired ability to secrete H^+ might be linked to the failure of a Cl^--HCO_3^- shuttle, thus impairing excretion of Cl^- and impairing reclamation of HCO_3^-.

4. Respiratory alkalosis (chronic)
   a. As a compensatory change to prolonged hypocapnia and alkalemia, there is increased renal retention of H^+ and thus reduced renal conservation of HCO_3^-.
   b. The fall in HCO_3^- is balanced by an increase in Cl^- and other anions that are not identified.[48]
5. Dehydration of sample (evaporation or sublimation): Exposure of serum or plasma to air may allow evaporation that results in hyperchloremia. This is especially true of air-conditioning systems that blow cool dry air over the sample processing or analysis areas. Sublimation of H_2O from frozen samples may result in hyperchloremia.

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 anion gap (see the Anion Gap section below).

V. Hypochloremia
   A. Hypochloremia typically occurs when there is hyponatremia (either true hyponatremia or pseudohyponatremia) or an increased serum [HCO_3^-] (as occurs with metabolic alkaloses). It may occur in a metabolic acidosis but the hypochloremia is more likely related to a concurrent hyponatremia than the acidotic state.
   B. Disorders and pathogeneses (Table 9.8)
      1. Cl^--deficit group (Cl^- loss > H_2O loss)

Table 9.8. Diseases 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^+ (see Na^+-deficit group in Table 9.4)
  - Metabolic alkaloses
    - [a]Loss or sequestration of HCl: vomiting, displaced abomasum, pyloric obstruction
    - [a]Bovine renal failure
    - Furosemide
    - Thiazide diuretics
  - Metabolic acidosis with an increased anion gap
    - [a]Ketoacidosis
    - [a]Lactic acidosis
    - Ingestion of foreign substance that generates anions: ethylene glycol
- H_2O-excess groups (with or without edema) (see same group in Table 9.4)
- Shifting of H_2O from ICF to ECF (see same group in Table 9.4)
- Shifting of Cl^- from intravascular to extravascular fluid (see similar Na^+-group in Table 9.4)

Note: Pseudohypochloremia may be caused by displacement of serum or plasma H_2O (see text).

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2. H_2O-excess group. (See Hyponatremia in Sodium Concentration, V.B.2 earlier in this chapter).
3. Shifting of H_2O from ICF to ECF. (See Hyponatremia in Sodium Concentration, V.B.3 earlier in this chapter.)
4. Shifting of Cl^- from intravascular to extravascular space. (See Hyponatremia in Sodium Concentration, V.B.5 earlier in this chapter.)

BICARBONATE (HCO_3^-) CONCENTRATION AND TOTAL CARBON DIOXIDE (tCO_2) CONCENTRATION

I. Physiologic processes
   A. HCO_3^- is a major buffer in the body that helps maintain blood pH at physiologic concentrations. HCO_3^- is produced from H_2O and CO_2 in cells that have carbonic anhydrase: erythrocytes, proximal renal tubular cells (luminal and intracellular), parietal cells of the gastric epithelium, and intercalated cells of the collecting tubules.
   B. In gastric or abomasal mucosal cells, HCl secretion is accomplished by the utilization of Cl^- from the ECF and by the generation of HCO_3^- (Fig. 9.5).
   C. 90% of filtered HCO_3^- is conserved in the proximal nephron (Fig. 9.6). This occurs during the process of H^+ secretion that is mediated through the Na^+-H^+ antiporter and is dependent on Na^+ resorption.
   D. Distal nephron
      1. HCO_3^- produced by the Type A intercalated cells of the collecting tubules 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 (Fig. 9.4).
      2. HCO_3^- can be excreted (when there is excess HCO_3^-) through a Cl^--HCO_3^- exchanger in Type B intercalated cells of the distal nephron.

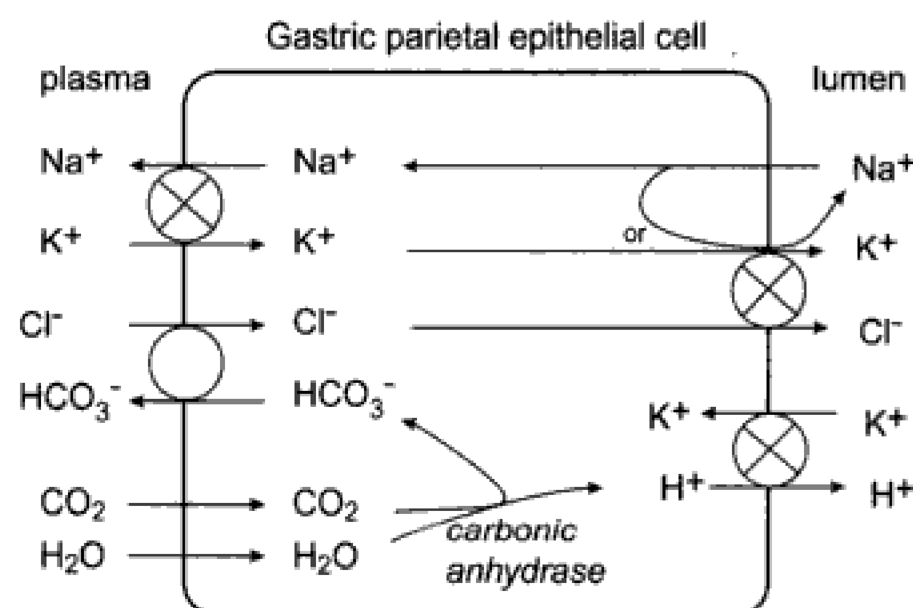

Fig. 9.5. Gastric or abomasal secretion of H^+ and Cl^-. (⊗ = ATPase pump, ○ = cotransporter or counter transporter [antiporter])

H^+ and HCO_3^- are formed 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^+ and K^+, but most Na^+ and K^+ ions are resorbed, thus leaving H^+ and Cl^- in the lumen. The major results of the process are gastric secretion of H^+ and Cl^-, a lower plasma [Cl^-], and higher plasma [HCO_3^-].

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increases. The magnitude of increase 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^-. Volume contraction also helps maintain the alkalosis by decreasing renal HCO_3^- excretion.

b. The primary processes that result in the loss of Cl^- (vomiting, sequestration, diuretics) may create the loss of H^+ and thus alkalosis.

IV. Decreased bicarbonate (HCO_3^-) concentration or total carbon dioxide (tCO_2) concentration

A. Decreased [HCO_3^-] is usually associated with metabolic acidosis, either primary or as compensation for respiratory alkalosis. The excess generation of H^+ or a loss of HCO_3^- typically produces a metabolic acidosis (see Fig. 10.1). The loss of HCO_3^- (or other body buffers) reduces the buffering capacity of the body and thus allows H^+ to accumulate.

B. Disorders and pathogeneses (Table 9.10)

1. Excess generation of H^+: If metabolic pathways generate sufficient acid 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^+.

a. Lactic acidosis occurs when cellular metabolism switches to anaerobic glycolysis.

b. Ketoacidosis occurs when there is excessive β-oxidation of triglycerides in hepatocytes.

c. Ingestion of certain compounds (e.g., ethylene glycol) creates an acidemia because the catabolism of the compound results in the generation of acid (see Table 9.12).

2. Decreased renal excretion of H^+: As with excess generation of acid, [HCO_3^-] decreases as it is used to buffer H^+ that accumulates in plasma.

a. Renal failure results in an 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 also produces an acidemia because of impaired urinary excretion of H^+.

Table 9.10. Diseases and conditions that cause decreased serum [HCO_3^-] or [tCO_2] (metabolic acidosis)

- Excess generation of H^+
  - [a]Lactic acidosis
  - [a]Ketoacidosis
  - Ingestion of certain compounds (see Table 9.12)
- Decreased renal excretion of H^+
  - [a]Renal failure
  - [a]Uroperitoneum or urinary tract obstruction
  - Distal renal tubular acidosis (type 1)
  - Hypoaldosteronism
- Increased HCO_3^- loss
  - [a]Alimentary losses: diarrhea, sequestration, or vomiting of pancreatic secretions
  - Renal losses: proximal renal tubular acidosis (type 2)
- Dilutional acidosis (rapid infusion of saline)

Note: Spurious decrease may occur due to aerobic sample handling.

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c. Distal renal tubular acidosis (type 1) occurs when there is decreased H^+ secretion by the distal tubules because of tubular disease. It may also occur with urinary tract obstruction and hyperkalemia.
d. Hypoaldosteronism (as seen with hypoadrenocorticism) may promote acidemia because H^+ (and K^+) secretion is promoted by aldosterone in the principal epithelial cells. Aldosterone also stimulates H^+-ATPase in the distal nephron and promotes H^+ secretion via the negative potential created by Na^+ resorption. Also, hyperkalemia inhibits NH_4^+ secretion. Thus, without aldosterone, there is less secretion of H^+. Hypoaldosteronism is called type 4 renal tubular acidosis.

3. Increased HCO_3^- loss
   a. Alimentary losses
      (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
      (1) In proximal renal tubular acidosis (type 2), there is a defect in HCO_3^- conservation in the proximal tubules. The defect may be due to abnormal Na^+ resorption or to the presence of a carbonic anhydrase inhibitor. The defect may be part of either an inherited or acquired Fanconi's syndrome. Besides proximal renal tubular acidosis, other findings in Fanconi's 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. In dogs, the disorder has been seen with hypocalcemia (due to hypoparathyroidism and hypovitaminosis D), streptozotocin and maleic acid treatments, and an overdose of amoxicillin.[52-55] Acquired proximal renal tubular acidosis was reported in a mare, but the cause was not determined.[56]
4. Dilutional acidosis may occur with rapid saline infusion: Rapid infusion of saline may decrease [HCO_3^-] by diluting ECF HCO_3^-. However, absolute change caused by dilution is expected to be minor and thus cause a minor change in blood pH.
5. *In vitro* loss of HCO_3^- from sample (see II.B above in this section)

ANION GAP

I. Definitions
   A. *Cation:* an atom or molecule with a positive charge. Monovalent cations have one positive charge, divalent cations have two.
   B. *Measured cation charge* (mC^+): the charge concentration of the major monovalent cations (Na^+ and K^+) whose serum activities or concentrations are directly measured. 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 concentration of all other cations in blood, including fCa^{2+}, fMg^{2+}, and cationic globulins, for which concentrations are not measured for the anion gap calculation
   D. *Total cation charge* (tC^+): total charge concentration of all cations in the blood ($tC^+ = mC^+ + uC^+$)

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E. *Anion:* an atom or molecule with a negative charge. Monovalent anions have one negative charge, divalent anions have two, trivalent anions (e.g., PO_4^{3-}) have three.

F. *Measured anion charge* (mA^-): the charge concentration of the major monovalent anions (Cl^- and HCO_3^-) whose serum activities or concentrations are measured. Because these ions are monovalent and measured as free ions, their ion concentrations are equivalent to their charge concentrations. [HCO_3^-] may be estimated from [tCO_2].

G. *Unmeasured anion charge* (uA^-): the charge concentration of all other anions in serum, including PO_4, albumin, anions of organic acids, and SO_4, for which concentrations are not measured for the anion gap calculation

H. *Total anion charge* (tA^-): total charge concentration of all anions in the blood ($tA^- = mA^- + uA^-$)

I. *Anion gap:* the difference (gap) in the charge concentrations between uA^- and uC^+ (anion gap = $uA^- - uC^+$). Anion gap can be calculated because this is also equal to the difference between mA^- and mC^+ concentrations (Eq. 9.3 and 9.4).

$$\text{Anion gap} = mC^+ - mA^- = ([Na^+]+[K^+]) - ([Cl^-]+[HCO_3^-]) \quad \textbf{(9.3.)}$$

II. Physiologic processes

A. Serum is always electrically neutral; i.e., total positive charges equal total negative charges. The major contributors to the electrical neutrality and their relative contributions are shown in Table 9.11. In the example, 150 of the 157 mmol/L of cation charge are from Na^+ and K^+ and 134 of the 157 mmol/L of anion charge are from Cl^- and HCO_3^-.

B. Anion gap formula derivation (Eq. 9.4)

$$\text{As serum is electrically neutral, } tC^+ = tA^-.$$
$$\text{As } tC^+ = mC^+ + uC^+ \text{ and } tA^- = mA^- + uA^-, \text{ then } mC^+ + uC^+ = mA^- + uA^- \quad \textbf{(9.4.)}$$
$$\text{As } mC^+ = [Na^+]+[K^+] \text{ and } mA^- = [Cl^-]+[HCO_3^-],$$
$$\text{then } ([Na^+]+[K^+]) + uC^+ = ([Cl^-]+[HCO_3^-]) + uA^-.$$
$$\text{Rearranging the equation, } uA^- - uC^+ = ([Na^+]+[K^+]) - ([Cl^-]+[HCO_3^-]).$$
$$\text{As anion gap} = uA^- - uC^+,$$
$$\text{then anion gap} = ([Na^+]+[K^+]) - ([Cl^-]+[HCO_3^-]).$$

Table 9.11. Cations and anions of serum in health (approximate charge concentrations provided to simplify concept)

Cations	mmol/L ion charge	Anions	mmol/L ion charge
Na^+	146	Cl^-	110
K^+	4.0	HCO_3^-	24
fCa^{2+}	5.0	Proteins	16[a]
fMg^{2+}	2.0	Organic anions	3.5
H^+	10^{-7}	PO_4	2.5
		SO_4	1.0
Total	157	Total	157

[a] Molar concentrations of proteins are not measured because of variations in molecular weights of proteins.

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5. With hyperphosphatemia, each 1 mg/dL increase in [PO_4] is associated with about a 0.6 mmol ion charge/L increase in uA^- and therefore in anion gap (1 mg/dL PO_4 = 0.323 mmol/L PO_4, at an average negative valence of about 1.8).

III. Analytical concepts
- A. Units: mmol ion charge/L (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)
- B. As with any calculated value, the anion gap will only be as accurate as the measured values. If needed, refer to prior sections on the individual electrolytes.
- C. The [HCO_3^-] used in the formula may be either [tCO_2] or a calculated [HCO_3^-]. Even with marked elevations in PCO_2 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.

IV. Increased anion gap
- A. Increased anion gaps typically are seen with certain metabolic acidoses (especially normochloremic), but it does not indicate which one. Hyperchloremic metabolic acidoses will typically not have an increased anion gap. Minor increases in the anion gap can be seen in nonacidotic disorders that cause hyperalbuminemia or reduced concentration of fCa^{2+} and fMg^{2+}.
- B. Disorders. Diseases and conditions that cause an increased anion gap are presented in Table 9.12.

V. Decreased anion gap
- A. A decreased anion gap has minimal to slight clinical significance, since it does not relate to specific pathologic states. Decreased anion gaps often occur when there is hypoalbuminemia (decreased uA^-). They may also occur with increased uC^+, but such changes are mild.
- B. Recognizing that hypoalbuminemia lowers the anion gap aids in the interpretation of anion gap values that are WRI or increased; i.e., the anion gap value would have been higher if the animal did not have hypoalbuminemia.

Table 9.12. Diseases and conditions that cause an increased anion gap

Metabolic acidoses
- [a]Lactic acidosis: increased lactate
- [a]Ketoacidosis: increased ketone bodies (β-hydroxybutyrate or acetoacetate)
- [a]Renal failure: increased PO_4, sulfate, or citrate
- Massive rhabdomyolysis: probably increased lactate and PO_4
- Ingestion of certain compounds
  - [a]Ethylene glycol (antifreeze): increased glycolate or oxalate
  - Methanol poisoning (antifreeze): formate
  - Paraldehyde (sedative or anesthetic): increased acetate and chloroacetate
  - Metaldehyde poisoning (snail bait)
  - Penicillin (very high doses)

Hyperalbuminemia (minor changes)

Note: Spurious increase in anion gap may occur when there is a decreased [HCO_3^-] caused by aerobic sample handling.

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Table 9.13. Diseases and conditions that cause a decreased anion gap

Decreases in unmeasured anion charge concentration (uA^-)
[a]Hypoalbuminemia
Increases in unmeasured cation charge concentration (uC^+)
Hypercalcemia (minor changes)
Hypermagnesemia (minor changes)
Multiple myeloma that is producing cationic proteins

Note: Spurious decrease in anion gap may occur when there is a pseudohyperchloremia caused by bromide.

C. Disorders. Diseases and conditions that cause a decreased anion gap are presented in Table 9.13.

VI. SID

A. Evaluation of SID assesses acid-base abnormalities in the context of abnormal concentrations of ions (strong cations and strong anions). SID concepts are similar to anion gap concepts but also consider other factors.

B. Complete explanation of SID is beyond the scope of this book, but major aspects are presented in Chapter 10.

OSMOLALITY AND OSMO. GAP

I. Definitions

A. *Osmolality:* the concentration of a solute expressed in moles of solute per ***kilogram of solvent*** (mol/kg). In clinical assessments, it is expressed as mosmol/kg.

B. *Osmolarity:* the concentration of a solute expressed in moles of solute per ***liter of solution*** (mol/L). In clinical assessments, it is expressed as mosmol/L. Serum osmolality does not equal serum osmolarity, because 1 L of normal serum will contain about 930 mL of H_2O (0.93 kg of H_2O) (assuming a total solids of 7 g/dL = 70 g/L = 0.07 kg/L).

C. *Osmole:* one mole of osmotically active particles. For a substance that does not dissociate in solution, 1 mole equals 1 osmole. For a substance that completely dissociates into two ions per mole, there are 2 osmoles of dissociated solute particles per 1 mole of undissociated substance. Most dissociable solutes do not dissociate completely.

D. *Mole:* the SI unit for the amount of substance present when there are 6.023×10^{23} identical particles (Avogadro's number) of it (based on the number of particles in 0.012 kg of carbon 12); 1 mole of a substance weighs its gram molecular weight; i.e., 1 mole of glucose weighs 180 g, 1 mole of Na weighs 23 g.

E. *Osmotic pressure:* the force required to counterbalance the force of osmotic solvent flow through a semipermeable membrane, such as cell membranes. It is also referred to as total osmotic pressure to differentiate it from colloidal osmotic pressure (see below).

F. *Osmosis:* the passage of solvent (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 $[H_2O]$)

G. *Osmometry:* any technique for measuring the osmolality of a solution

H. *Osmometer:* a device or instrument that measures the osmolality of a solution

I. *Tonicity:* the *effective* osmolality of a solution; i.e., that solute concentration that can contribute to movement of H_2O across a semipermeable membrane

J. *Solute:* a substance dissolved in a solvent

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K. *Colloidal osmotic pressure (oncotic pressure):* the osmotic pressure exerted by colloidal particles suspended in a solvent at a capillary membrane. Colloidal particles are macromolecules that are too small (1 nm to 1 μm) to settle out due to gravity. Most oncotic pressure of plasma is caused by plasma proteins (about 80% albumin, 20% globulins); part of the colloidal 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.

II. Physiologic processes
  A. As stated above, osmolality is the concentration of solutes per kilogram of solvent, and it is dependent on the number of molecules or ions in the solution. The major contributors to serum osmolality and their relative contributions are shown in Table 9.14.
  B. Major concepts of relative contributions of solutes to serum osmolality
    1. Na^+ is the major solute in serum. In fact, about half of the solute (moles, not mass) in serum are Na^+ ions.
    2. Cl^- runs a close second. Because Cl^- frequently follows sodium, changes in $[Na^+]$ are frequently accompanied by changes in $[Cl^-]$, which approximately doubles the changes in osmolality.
    3. At physiologic concentrations, urea and glucose are small contributors to total osmolality. However, marked azotemia or hyperglycemia will cause hyperosmolality.
    4. Protein contributes very little to osmolality (< 1 mosmol/kg). Protein molecules are relatively very large but extremely rare (compared to electrolytes, urea, and glucose).
    5. Hypothalamic osmoreceptors are sensitive to increases and decreases in effective plasma osmolality (tonicity).
      a. If there is an increased effective osmolality, ADH is released to stimulate the renal collecting tubules 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 plasma solute concentration.

Table 9.14. Solutes that contribute to serum osmolarity (approximate concentrations provided to simplify concept)

Solute	Measured concentration	Factor to convert to mosmol/L	Contribution to osmolarity	Contribution to total osmolarity in serum of healthy animal
Na^+	146 mmol/L	× 1	146 mosmol/L	281 mosmol/L 94% of total solute
K^+	4 mmol/L	× 1	4 mosmol/L	
Cl^-	107 mmol/L	× 1	107 mosmol/L	
HCO_3^-	24 mmol/L	× 1	24 mosmol/L	
UN	20 mg/dL	÷ 2.8	7 mosmol/L	12 mosmol/L 4% of total solute
Glucose	100 mg/dL	÷ 18	5.5 mosmol/L	
PO_4	4 mg/dL	× 0.6	2.4 mosmol/L	6 mosmol/L 2% of total solute
tCa	10 mg/dL	× 0.25	2.5 mosmol/L	
Mg^{2+}	1 mmol/L	× 1	1.0 mosmol/L	
Protein	6 g/dL	Varies with protein	< 1.0 mosmol/L	No significant contribution to osmolarity
Total			300.4 mosmol/L	

The data in the table illustrate the contributions of common serum solutes to total solute concentration. The data are expressed as osmolarities because the measured concentrations of individual solutes are routinely reported on a */L* basis and not on a */kg H_2O* basis. Assuming total solids occupt 7% of plasma volume and thus H_2O occupies 93% of plasma volume, osmolarity is converted to osmolality as follows: osmolarity ÷0.93 = osmolality.

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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.
3. Osm_m indicates the total concentration of solutes in the serum, but it does not indicate which solutes are present.
4. Serum is the required sample for measuring osmolality; addition of anticoagulants for plasma samples would be adding solute to the plasma. The stability of a serum osmolality will depend 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. 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 allows us to estimate 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; most plasma solids are proteins.
   b. The concentrations of some plasma solutes are not measured and thus their contributions to total osmolality are not included in calculations.
   c. Some plasma solutes are not completely dissociated (e.g., minute amounts of Na^+ and Cl^- are present in plasma as NaCl).
2. At least 14 formulas have been used to calculate an estimate (Osm_c) of the true osmolality (Osm_m) of serum.[58] Each formula includes measured values for some of the major solutes contributing to osmolality, and constants or factors to estimate the effects of the other solutes. The formulas vary because:
   a. Different investigators attempted to estimate true osmolality 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 true osmolality.
   c. The dissociation of ionic compounds may vary among species.
   d. The conversion of serum osmolarity to serum osmolality is imperfect.
3. Ideally, each laboratory would determine a best-fit equation to predict Osm_m for that laboratory's assays. However, most use one of four equations (Eq. 9.6a-d). Other formulas add a constant (e.g., 8.6) to estimate the contribution of solutes other than Na^+, K^+, Cl^-, HCO_3^-, urea, and glucose.

In all formulas, Na^+ and K^+ concentrations are in mmol / L or mEq / L.

If UN and glucose concentrations are in mg / dL, then

$$Osm_c = 1.86\ ([Na^+] + [K^+]) + \frac{[UN]}{2.8} + \frac{[glucose]}{18} \quad \textbf{(9.6.a.)}$$

$$Osm_c = 2\ [Na^+] + \frac{[UN]}{3} + \frac{[glucose]}{20} \quad \textbf{(9.6.b.)}$$

If UN and glucose concentrations are in mmol / L, then

$$Osm_c = 1.86\ ([Na^+] + [K^+]) + [urea] + [glucose] \quad \textbf{(9.6.c.)}$$

$$Osm_c = 2\ [Na^+] + [urea] + [glucose] \quad \textbf{(9.6.d.)}$$

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4. What are the components of the equations?
   a. $1.86 \times ([Na^+] + [K^+])$ or $2 \times [Na^+]$: an estimate of the osmolality due to the major four electrolytes; i.e., the sum of Na^+, K^+, Cl^-, and HCO_3^- concentrations
      (1) Because serum must remain electrically neutral, increased concentrations of other anions will be associated with lower $[Cl^-]$ or $[HCO_3^-]$ if cation concentrations remain constant. Thus, the expression estimates the osmolality due to electrolytes even when there is an increased anion gap.
      (2) The inclusion of K^+ tends to yield a more accurate estimate of osmolality, especially if there is hyponatremia and hyperkalemia. However, because serum $[K^+]$ cannot change much (± 2 mmol/L) without serious medical consequences, major changes in serum $[K^+]$ cause only minor changes in total serum osmolality.
   b. $[UN] \div 2.8$: conversion of [UN] from mg/dL to mmol/L of urea (M_r of UN is 28 and there are 10 dL in 1 L)
   c. $[UN] \div 3$: approximate conversion of [UN] from mg/dL to mmol/L of urea
   d. $[glucose] \div 18$: conversion of [glucose] from mg/dL to mmol/L (M_r of glucose is 180 and there are 10 dL in 1 L)
   e. $[glucose] \div 20$: approximate conversion of [glucose] from mg/dL to mmol/L
5. *An Osm_c is of little value by itself;* it is simply an estimate of the osmolality due to commonly measured solute concentrations. If a measured solute concentration (i.e., Na^+, urea, glucose) is increased, then the Osm_c will be increased proportionately and it may or may not estimate the true serum osmolality.

C. Osmo. gap
1. There is not a correct unit for the osmo. gap because the Osm_m is expressed as mosmol/kg (molality) and the Osm_c is expressed as mosmol/L (molarity). Since neither *osmolar gap* nor *osmolal gap* is a correct term, the term *osmo. gap* will be used.
2. Osmo. gap formula (Eq. 9.7)
   a. Osm_m represents the total solute concentration in the sample. Osm_c estimates the solute concentration using some combination of Na^+, K^+, urea, and glucose.
   b. If the formula used for Osm_c is optimized to match Osm_m values, the osmo. gap reference interval would be near zero.

$$\text{Osmo. gap} = Osm_m - Osm_c \qquad \textbf{(9.7.)}$$

IV. Evaluation of serum osmolality and osmo. gap. The major concepts are shown in Fig. 9.8.

V. Interpretation of osmolality data (Table 9.15)

A. Serum hyperosmolality and osmo. gap WRI: An increased Osm_m indicates there is an increased concentration of one or more solutes. Since the osmo. 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^+, urea, or glucose, then the increased Osm_m is due to increased Na^+, urea, or glucose concentrations.

B. Serum hyperosmolality and increased osmo. gap: An increased Osm_m indicates there is an increased concentration of one or more solutes. Because the osmo. 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 osmo. gap are associated with poisonings by nonelectrolytes (e.g., ethylene glycol, methanol, paraldehyde) or intravenous administration of mannitol or radiographic contrast media. The increased osmo. gap is probably due to a nonionic solute other than urea or glucose for the following reasons:

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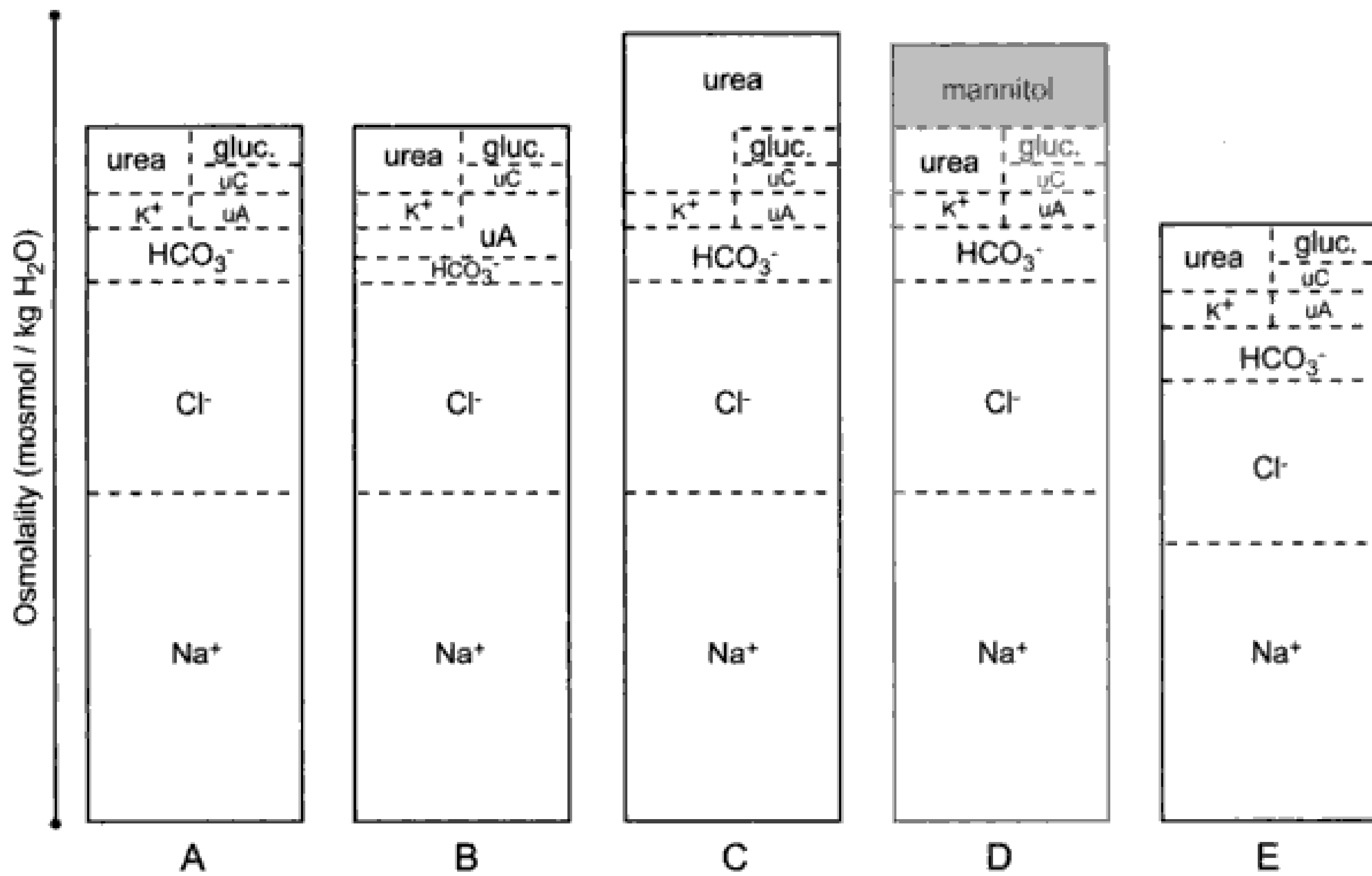

Fig. 9.8. Bar diagrams of serum osmolality concepts.

uC = [fCa^{2+}] + [Mg^{2+}]; uA = [PO_4] + [SO_4] + [anions of organic acids], all in mmol/L units. Proteins are not included because they do not contribute significantly to osmolality. Solutes that contribute to the osmo. gap are within the grey-shaded area.

A. In the healthy animal, nearly all nonprotein solutes are monovalent electrolytes except for urea and glucose; osmo. gap should be near 0 if an appropriate formula for Osm_c is used.

B. In a normochloremic metabolic acidosis (therefore low [HCO_3^-]), the osmo. gap is not increased because the 1.86 ([Na^+] + [K^+]) should account for the sum of all cations and anions. Because electrical neutrality must be maintained, increases in uA must be accompanied by increases in cations, decreases in other anions, or both. In any case, the 1.86 (Na^+ + K^+) should account for the sum of all cations and anions.

C. When hyperosmolality is due to azotemia, the osmo. gap is not increased because both the Osm_m and Osm_c are increased proportionately by the higher urea concentration. The same concept applies for hyperosmolality due to hyperglycemia or hypernatremia. If azotemia and hyperphosphatemia are present, the PO_4 ions will contribute to an increased anion gap but they will not contribute to an osmo. gap (see Table 9.15).

D. When hyperosmolality is due to an abnormal solute (e.g., mannitol), the osmo. gap is increased because the exogenous solute increases the Osm_m but is not included in the Osm_c formula.

E. When hypoosmolality is due to hyponatremia and hypochloremia, the osmo. gap is not changed because the Osm_m and Osm_c are decreased proportionately.

1. Increases in [Na^+], [K^+], or anions (Cl^-, HCO_3^-, or others) would result in an 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 the "1.86 ([Na^+] + [K^+])" and "2 [Na^+]" expressions estimate the concentration of all ions, assuming that Eqs. 9.6.a and 9.6.b provide reliable estimates of the serum osmolality. Because the expressions do not account for biologic variation in ion concentrations (within an animal, between animals, or between species), we must remember that the expressions only provide estimates.

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Table 9.15. Interpretation of abnormal serum osmolality or increased osmo. gaps

Osm_m	Osmo. gap	Abnormal [solute]	Disorders, conditions
ᵃIncreased	WRI	Increases in Na^+, urea, or glucose concentrations	See Hypernatremia section See Azotemia and Uremia (Chap. 8) See Hyperglycemia (Chap. 14)
Increased	Increased	Increased concentration of nonionic compound other than urea or glucose	Mannitol infusion (intravenous) Radiographic contrast media (intravenous) Ethanol, methanol, ethylene glycol
ᵃDecreased	WRI	Indicates a true hyponatremia	See Hyponatremia section

Note: A spurious increase in osmo. gap will occur if there is a pseudohyponatremia (see Hyponatremia section).

2. As previously explained in the Osm_c section, the 1.86 ($[Na^+]$ + $[K^+]$) and 2 $[Na^+]$ expressions will represent total concentration of ions because serum must remain electrically neutral. Even when an anion gap is increased because of increased concentrations of anions other than Cl^- or HCO_3^-, the expressions will estimate ionic contributions to osmolality because there will be either proportionally lower $[Cl^-]$ and/or $[HCO_3^-]$ (therefore no net change in Osm_m or Osm_c) or proportionally increased $[Na^+]$ and/or $[K^+]$ (therefore increases in both Osm_m and Osm_c) to maintain electrical neutrality. In either case, the expressions represent total ion concentrations and osmo. gap will not be due to anions that create an increased anion gap.
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 of $[fCa^{2+}]$ or $[fMg^{2+}]$ of 2–3 mmol/L would create pathologic states but increase the serum osmolality by no more than 4-6 mosmol/kg (increase in cation charge concentration could be matched by increase in anion charge concentration, or a reduction in $[Na^+]$ and/or $[K^+]$). A change in osmo. gap of 4–6 units typically would be considered clinically insignificant because it could represent physiologic or analytical variation.
4. Even though the increased osmo. gap will be due to an increased concentration of a nonionic solute, there can be a concurrent increase in anions to create an anion gap.[59] Methanol can contribute to an osmo. gap and its metabolite (formate) can contribute to an anion gap. Ethylene glycol can contribute to an osmo. gap and its metabolites (glycolate or oxalate) can contribute to an anion gap. In ketoacidosis, acetone might contribute to osmo. gap but acetoacetate and β-hydroxybutyrate contribute to the anion gap.

C. Serum hypoosmolality and osmo. gap WRI: A decreased Osm_m indicates there is a decreased concentration of one or more solutes. Because the osmo. gap is WRI, there is a proportionate decrease in Osm_c and Osm_m and thus there must be decreased concentrations of Na^+, UN, or glucose. As UN and glucose contribute about 7 and 5 mosmol/kg, respectively, in health, and a marked decrease in either (e.g., from 7 to 3 or 5 to 2) causes only a minor decrease in total osmolality, any clinically significant hypoosmolality will be due to hyponatremia.

D. If there is a spurious hyponatremia, the Osm_m will be an accurate value because lipids or proteins do not affect the $[Na^+]$ in plasma H_2O. However, the Osm_c will be decreased because of the spuriously low $[Na^+]$. Therefore, the osmo. gap will be increased.

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Chapter 10

BLOOD GASES, BLOOD pH, AND STRONG ION DIFFERENCE

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Table 10.1. Abbreviations and symbols in Chapter 10

»	symbol in tables for relatively common disease or condition
[x]	concentration of x; x = analyte
2,3–DPG	2,3–diphosphoglycerate
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
$CO_{2(g)}$	gaseous carbon dioxide
CO_3^{2-}	carbonate
COHgb	carboxyhemoglobin
ECF	extracellular fluid
fCa^{2+}	free ionized calcium
FiO_2	fractional concentration of O_2 in inspired gas
fMg^{2+}	free ionized magnesium
H_2CO_3	carbonic acid
H_2O	water
$H_2PO_4^-$	dihydrogen phosphate
HCO_3^-	bicarbonate
Hct	hematocrit
Hgb	hemoglobin
HPO_4^{2-}	hydrogen phosphate
ICF	intracellular fluid
MetHgb	methemoglobin
NH_3	ammonia
$O_{2(g)}$	gaseous oxygen
$O_2Hgb\%$	fractional hemoglobin saturation (also called oxyhemoglobin fraction and oxyhemoglobin percentage)
P_aCO_2	partial pressure of carbon dioxide in arterial blood
P_aO_2	partial pressure of oxygen in arterial blood
PCO_2	partial pressure of carbon dioxide
PO_2	partial pressure of oxygen
PO_4	phosphate (all forms)
PO_4^{3-}	phosphate
P_vCO_2	partial pressure of carbon dioxide in venous blood
P_vO_2	partial pressure of oxygen in venous blood
S_aO_2	percent Hgb saturation with O_2 in arterial blood
SID	strong ion difference
$SID_{approximate}$	approximate strong ion difference
$SID_{measured}$	measured strong ion difference
SID_{true}	true strong ion difference
SO_2	percent Hgb saturation with O_2
SO_4	sulfate (all forms)
SO_4^{3-}	sulfate
SpO_2	percent Hgb saturation with O_2 of arterial blood by pulse oximetry, "p" is for "pulse" oximetry
tCO_2	total carbon dioxide
UN	urea nitrogen
uSA^-	unidentified strong anion
WRI	within reference interval

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DEFINITIONS

Definitions of important terms in this chapter:

Acidemia: a decreased blood pH (increased $[H^+]$)
Alkalemia: an increased blood pH (decreased $[H^+]$)
Acidosis: a condition in which acidemia tends to occur; animal may not be acidemic because of compensating mechanisms or because of the width of the reference interval
Alkalosis: a condition in which alkalemia tends to occur; animal may not be alkalemic because of compensating mechanisms or because of the width of the reference interval
Hypercapnia: excess CO_2 in blood (increased P_{CO_2})
Hypocapnia: deficiency of CO_2 in blood (decreased P_{CO_2})
Hypoxemia: deficiency of dissolved O_2 in blood (decreased P_{O_2})

PHYSIOLOGIC PROCESSES

I. Several respiratory and nonrespiratory processes help maintain $[H^+]$ 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, NH_3, sulfates, Hgb, and other proteins such as albumin). Of the total buffering capacity in health, HCO_3^- contributes over 20 mmol/L, whereas the nonbicarbonate buffers contribute less than 10 mmol/L.[1]

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 relationship of $[H^+]$, $[HCO_3^-]$, and P_{CO_2} in normal blood at 37°C can be expressed with the Henderson-Hasselbalch equation or a nonlogarithmic version (Eq. 10.1.a). The $[H_2CO_3]$ is calculated from a measured P_{CO_2} value based on the solubility coefficient of CO_2 in an aqueous solution (Eq. 10.1.b).

$$pH = 6.1 + \log\frac{[HCO_3^-]}{[H_2CO_3]} \text{ or } [H^+] = 24 \times \frac{P_{CO_2}}{[HCO_3^-]}; \quad \textbf{(10.1.a.)}$$

$$P_{CO_2} \times 0.03 = [H_2CO_3] \quad \textbf{(10.1.b.)}$$

$$[H^+] \text{ in nmol/L; } [HCO_3^-] \text{ \& } [H_2CO_3] \text{ in mmol/L; } P_{CO_2} \text{ in mmHg}$$

   B. As seen in the nonlogarithmic equation, $[H^+]$ is clearly related to the ratio of P_{CO_2} to $[HCO_3^-]$. As the ratio increases, the animal becomes acidemic. As the ratio decreases, the animal becomes alkalemic.

III. Pulmonary functions related to acid-base status
   A. Expiration of $CO_{2(g)}$ results in the elimination of H^+ (Fig. 10.1A).
      1. Because blood $[H^+]$ is very low compared to $[HCO_3^-]$ (ratio ≈ 1:600,000), this process does not lower $[HCO_3^-]$ unless there is excessive generation of H^+.
      2. It may be helpful to consider the bicarbonate system going through H_2CO_3 (Eq. 10.2.a), but the actual reaction catalyzed by carbonic anhydrase involves the dissociation of H_2O, release of H^+, reaction of OH^- with CO_2 to form HCO_3^- and without a H_2CO_3 intermediate (Eq. 10.2.b). The reaction is reversible.

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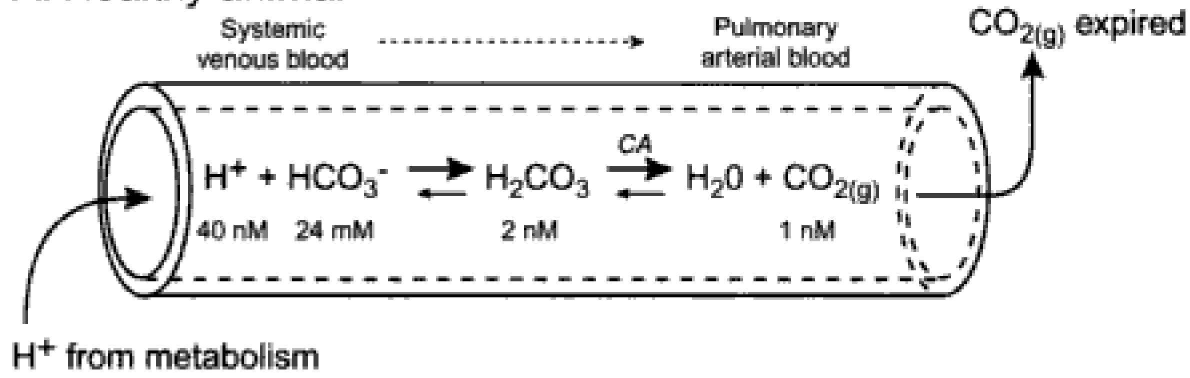
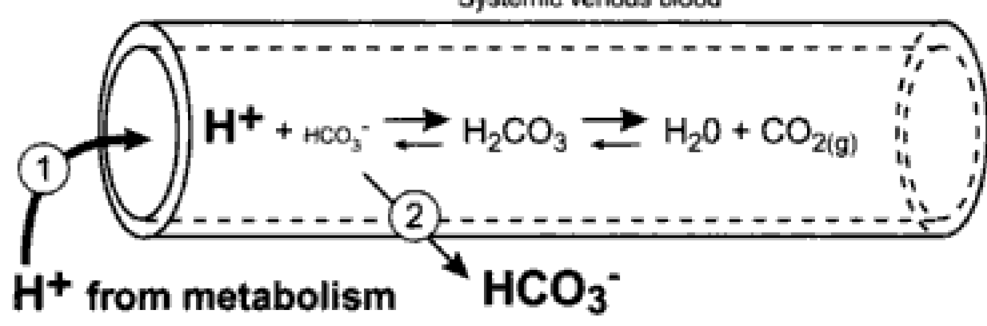
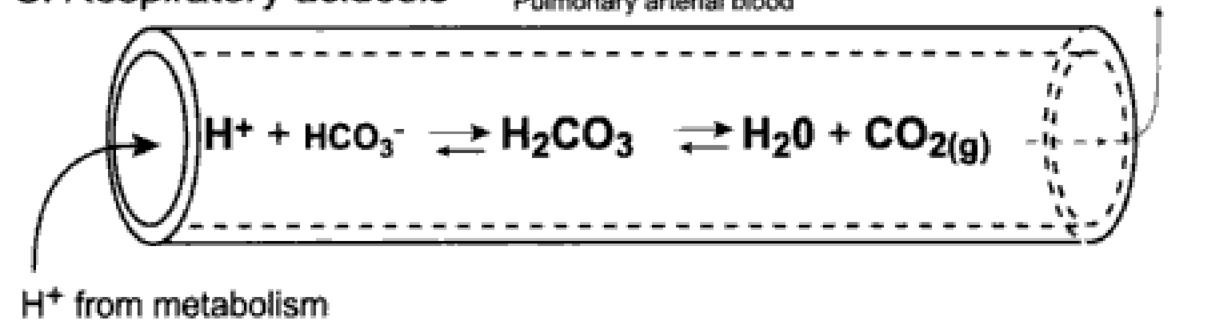
A. Healthy animal



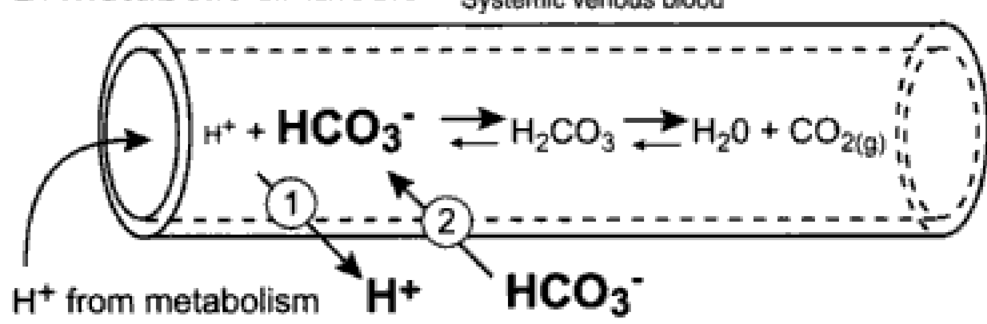
B. Metabolic acidosis



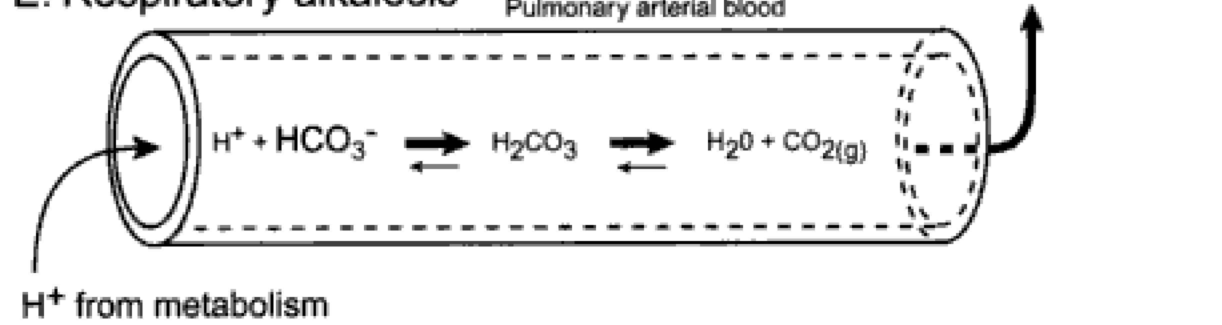
C. Respiratory acidosis



D. Metabolic alkalosis



E. Respiratory alkalosis



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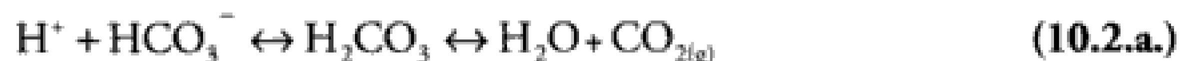

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow H_2O + CO_{2(g)} \quad \text{(10.2.a.)}$$

$$H^+ + HCO_3^- \xleftrightarrow{\text{carbonic anhydrase}} H_2O + CO_{2(g)} \quad \text{(10.2.b.)}$$

B. Hyperventilation increases expiration of CO_2 and tends to cause alkalemia; hypoventilation decreases expiration of CO_2 and tends to cause acidemia. Two types of chemoreceptors control respiration.
   1. Central chemoreceptors (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 $[H^+]$).

IV. Renal functions related to acid-base balance
   A. Kidneys have key roles in maintaining acid-base status (see Chaps. 8 and 9).
   B. Their major functions that pertain to acid-base status in health are to excrete H^+ (directly or by incorporation into NH_4^+, $H_2PO_3^-$, or SO_4^{2-}) and to conserve HCO_3^-.
   C. Hormonal and other factors that alter Na^+, Cl^-, and K^+ excretion will typically influence renal excretion of H^+ and HCO_3^-.

Fig. 10.1. Schematic representation of basic concepts of bicarbonate buffering system in health and in acid-base disorders.

Respiratory disorders involve removal of CO_2 from pulmonary arterial (capillary) blood. Metabolic disorders result in abnormal concentrations of H^+ and HCO_3^- in systemic venous blood.

A. In health, H^+ from metabolism is buffered by HCO_3^- to form H_2CO_3, which dissociates to H_2O and $CO_{2(g)}$. The $CO_{2(g)}$ is expired via the respiratory system. In the presence of carbonic anhydrase (*CA*), the reactions are reversible but the net flow is to the right (toward $CO_{2(g)}$ expiration). The approximate molar concentrations of H^+, HCO_3^-, H_2CO_3, and dissolved $CO_{2(g)}$ in plasma show that there is large excess of HCO_3^- available to buffer H^+.

B. In a metabolic acidosis, acidosis occurs because of one of two basic processes; without compensation, P_{CO_2} remains WRI. However, ↑ $[H^+]$ will stimulate respiration and result in increased removal of CO_2 from pulmonary blood and thus a ↓ P_{CO_2}.
   1. Excess H^+ accumulates because of increased production of organic acids or decreased renal excretion of H^+. The excess H^+ drives the equation to the right and thus leads to consumption of HCO_3^-.
   2. Excess loss of HCO_3^- via alimentary or urinary system reduces the buffering capacity and allows H^+ to accumulate.

C. In a respiratory acidosis, hypoventilation causes reduced expiration of $CO_{2(g)}$, which leads to an ↑ P_aCO_2 and an ↑ $[H^+]$ (↓ pH); without compensation, $[HCO_3^-]$ is insignificantly increased and remains WRI. Given time, the kidneys will compensate for acidemia and conserve HCO_3^-.

D. In a metabolic alkalosis, alkalosis occurs because of one of two basic processes; without compensation, P_{CO_2} remains WRI.
   1. Excess H^+ is lost via gastric or renal secretion; the secretion of H^+ results in a generation of HCO_3^- that accumulates in plasma.
   2. Excess HCO_3^- is formed, conserved, or administered and thus results in more removal of H^+ from blood and thus an alkalemia.

E. In a respiratory alkalosis, hyperventilation causes excessive expiration of $CO_{2(g)}$, which leads to a ↓ P_aCO_2 and a ↓ $[H^+]$ (↑ pH); without compensation, $[HCO_3^-]$ remains WRI.

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ANALYTICAL CONCEPTS

I. Blood gas instruments
   A. Blood gas instruments measure the $[H^+]$, P_{O_2}, and P_{CO_2} in blood.
      1. Selective membranes allow only H^+, $O_{2(g)}$, or $CO_{2(g)}$ to pass from the blood to cause reactions with specific electrodes. In the P_{CO_2} system, the CO_2 reacts in a bicarbonate buffer to change the pH; the change in pH is detected by a pH (H^+-selective) electrode.
      2. The membranes also protect the electrodes from proteins and other substances in the blood. Maintenance of blood gas instruments involves maintenance (cleaning, replacement) of the membranes.
   B. Point-of-care testing: Small hand-held or portable chemical analyzers that use a disposable cartridge that contain ion-selective electrodes can measure blood gas values (pH, P_{CO_2}, P_{O_2}), common blood analyte concentrations (Na^+, K^+, Cl^-, urea, glucose, fCa^{2+}, Hgb), and calculate several concentrations or values (HCO_3^-, tCO_2, anion gap, S_{O_2}, BE_{ECF}, BE_B, Hct).
   C. Calculated acid-base and blood gas values: The following values can be calculated from the measured pH, P_{CO_2}, and P_{O_2} using other known factors (e.g., body temperature, [Hgb]):
      1. $[HCO_3^-]_{actual}$: calculated via the Henderson-Hasselbalch equation using the measured pH and measured P_{CO_2} (Eq. 10.1.a)
      2. $[tCO_2]$: calculated from the calculated $[HCO_3^-]_{actual}$ and measured P_{CO_2} (Eq. 10.3); the P_{CO_2} is multiplied by 0.03 (a solubility coefficient) to calculate the amount of dissolved CO_2 in the blood sample.

$$[tCO_2] = [HCO_3^-] + [H_2CO_3] = [HCO_3^-] + (P_{CO_2} \times 0.03) \qquad \mathbf{(10.3.)}$$

      3. $[HCO_3^-]_{standard}$
         a. $[HCO_3^-]_{standard}$ is the $[HCO_3^-]$ in plasma when fully oxygenated blood from a "normal" animal is equilibrated at 37°C and P_{CO_2} of 40 mmHg. Or, a calculated $[HCO_3^-]_{standard}$ is an estimate of the $[HCO_3^-]$ in the sample if the P_{CO_2} was 40 mmHg.
         b. The $[HCO_3^-]_{standard}$ is a better estimate of buffer base in a blood sample because it is independent of changes in P_{CO_2}, whereas a $[HCO_3^-]_{actual}$ is dependent on P_{CO_2} values (e.g., when P_{CO_2} increases, $[HCO_3^-]$ increases in plasma).[2]
      4. BE_{ECF} or BE_P (in mmol/L)[1]
         a. BE_{ECF} value is the amount of strong acid (mmol/L) that is needed to titrate extracellular fluid (or plasma) to a pH of 7.4 if the P_{CO_2} is 40 mmHg at 37°C.
         b. The calculation uses the measured pH, calculated $[HCO_3^-]$, an assumed normal $[HCO_3^-]$ of 24.5 mmol/L, and an assumed normal plasma protein concentration of 7.2 g/dL.
         c. Changes in BE_{ECF}
            (1) A positive BE_{ECF} indicates there is an excess of plasma buffer (e.g., HCO_3^-, proteins) and thus a metabolic alkalosis.
            (2) A negative BE_{ECF} indicates there is a deficit of plasma buffer (e.g., HCO_3^-, proteins) and thus a metabolic acidosis.
            (3) If blood pH changes because of a change in P_{CO_2} but the $[HCO_3^-]$ is WRI, then the BE_{ECF} will remain WRI.

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5. BE_B (in mmol/L)
   a. BE_B value is the amount of strong acid (mmol/L) that is needed to titrate 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 erythrocytes to the buffer base (most from Hgb, but also from 2,3-DPG).
   c. Positive and negative BE_B values provide essentially the same information about the patient as the BE_{ECF}. The BE_B values should approach the BE_{ECF} as an animal becomes more anemic.
   d. BE_B can also be derived via a Siggaard-Andersen alignment nomogram.
6. Buffer base (or total buffer base)
   a. Buffer base is the sum of "buffer ions" of blood, including bicarbonate, Hgb, and plasma proteins.
   b. Buffer base is related to base excess as follows: buffer $base_{actual}$ = buffer $base_{normal}$ + base excess. Buffer $base_{actual}$ is the calculated sum of the "buffer ions" in the patient's sample; buffer $base_{normal}$ is the expected sum of the "buffer ions" in a healthy human; and base excess (if positive) is the amount of excess "buffer ions" compared to normal.
7. SO_2 (percentage)
   a. The SO_2 is the amount of O_2 in blood divided by the O_2 carrying capacity of blood (expressed as a percentage).
   b. The SO_2 is primarily dependent on the PO_2 as described by the sigmoid oxygen-Hgb dissociation curve (Fig. 10.2). At a P_aO_2 of 100 mmHg, the SO_2 is expected to be about 97%. Acidemia, increased erythrocyte [2,3-DPG], hyperthermia, and hypercarbia shift the dissociation curve to the right (SO_2 decreases for a given PO_2).
   c. When the SO_2 is calculated using measured pH and PO_2 values and:
      (1) the pH is constant but the PO_2 decreases, then SO_2 decreases.
      (2) 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.

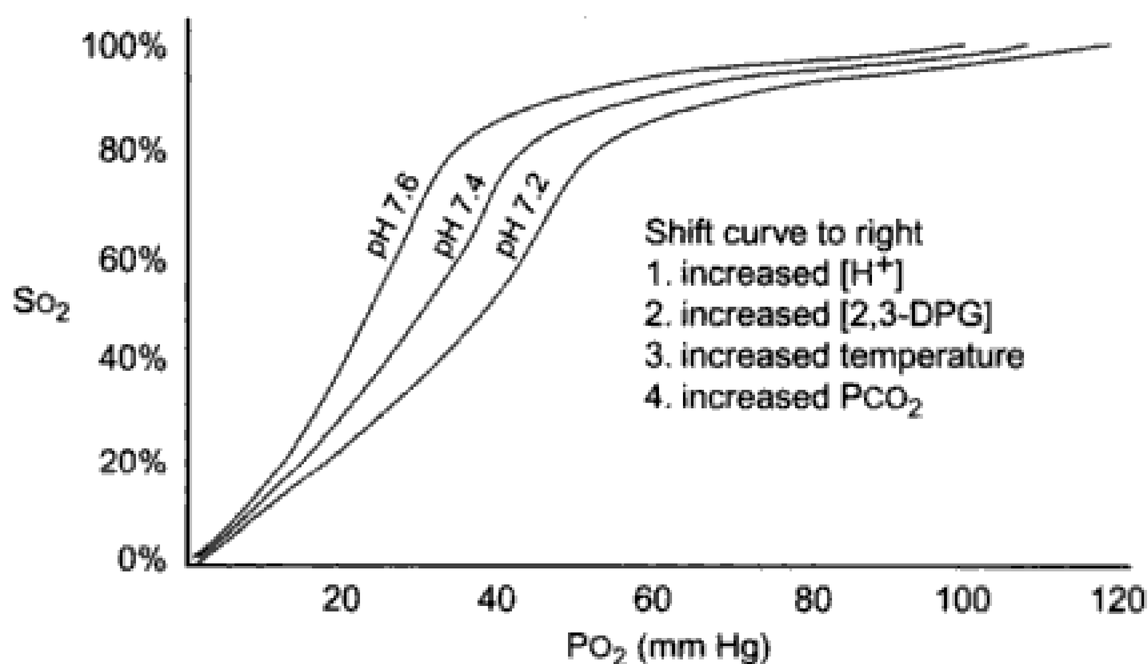

Fig. 10.2. Schematic drawing of oxygen-hemoglobin dissociation curve.
Increased [H^+], increased erythrocyte [2,3-DPG], hyperthermia, and hypercarbia will shift the curve to the right. If PO_2 stays constant, shifting the curve to the right will result in a lower SO_2. Also note that when PO_2 values are greater than 100 mm Hg, a shifting of the curve will result in very little change in SO_2 because the SO_2 will be near 100%.

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the measured absorbance values, a ratio can be calculated that reflects the S_aO_2 (Eq. 10.4.a).

$$S_aO_2 = \frac{[O_2Hgb]}{[O_2Hgb]+[RHgb]} \times 100 \quad \textbf{(10.4.a.)}$$

$$O_2Hgb\% = \frac{[O_2Hgb]}{[O_2Hgb]+[RHgb]+[COHgb]+[MetHgb]} \times 100 \quad \textbf{(10.4.b.)}$$

O_2Hgb (oxyhemoglobin), RHgb (reduced hemoglobin), COHgb (carboxyhemoglobin), MetHgb (methemoglobin)

3. The interference caused by other light-absorbing substances in the tissues is removed by measuring the light while arterial blood *pulses* through the tissue. In pulse oximetry, it is assumed that the changes in absorbance values occur when oxygen-rich blood pulsates through the tissue.
4. COHgb and MetHgb also absorb light at 660 and 940 nm and thus they do interfere with measurements if present (see Hypoxemia section below).

III. Sample for blood gas and pH analysis

A. Heparinized whole blood: 0.05 to 0.1 mL of heparin (1000 units/mL) per mL of blood, or 50–100 units/mL of blood

1. Arterial blood is the preferred sample for all blood gas and pH analyses and is required for the assessment of oxygenation of blood or pulmonary function.
2. Venous blood may give adequate results for assessment of metabolic disorders but will have lower pH, higher P_{CO_2}, and lower P_{O_2} values. The P_{O_2} of venous blood is not an accurate reflection of pulmonary function. The source of the venous blood may affect the P_{CO_2} value. Venous blood from a hypoxic leg will have a different P_{CO_2} value than venous blood from a well-perfused head.
3. Blood may be collected in either glass or quality plastic syringes. The needle must be sealed with cork or rubber immediately after collection to prevent exposure to air. Air should not be in the syringe during or after the collection of a sample.
4. Optimally, a heparinized blood sample should be tested (point-of-care) or transported to the laboratory immediately after collection. If not possible, acceptable blood gas and pH results can be obtained if the heparinized blood is immersed in an ice bath and analyzed within 1 hr. Placement of the sample in a refrigerator will not cool the sample as quickly; do not place a sample in a freezer because erythrocytes will lyse.

B. Erroneous blood gas and pH values due to poor sample collection or handling

1. Exposure to air (including air bubbles) or excess heparin in the sample[6]
   a. The P_{O_2} and P_{CO_2} values for arterial blood and room air are different (Table 10.3). The P_{O_2} and P_{CO_2} of heparin will be the same as room air if heparin is exposed to air.

Table 10.3. P_{O_2} and P_{CO_2} values in air and blood (samples from healthy animal inspiring room air)

		Room air	Arterial blood	Venous blood
P_{O_2}	mmHg	≈ 150 mm Hg	≈ 90 mmHg	≈ 40 mmHg
P_{CO_2}	mmHg	< 1 mmHg	≈ 40 mmHg	≈ 46 mmHg

Source: Muir WW, III, Hubbell JAE: 1989. Acid-base balance and blood gases. In: *Handbook of Veterinary Anesthesia,* 191-201. St. Louis: C.V. Mosby Company.

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Table 10.4. Diseases and conditions that cause respiratory acidosis (hypoventilation)

Inhibition or dysfunction of medullary respiratory center
Drugs: anesthetics, sedatives, narcotics
Brain stem disease (trauma, infection, neoplasia, etc.)
Alkalemia due to a metabolic alkalosis
Inhibition or dysfunction of respiratory muscles (diaphragm, chest wall): tick paralysis, tetanus, botulism, myasthenia gravis, hypokalemia, succinylcholine
Upper airway dysfunction: foreign body, vomitus
Impaired gas exchange at pulmonary capillaries
Pulmonary disease: infection, allergy, neoplasia, hyaline membrane disease in neonates
Restrictive disease: pneumothorax, pleural effusions, diaphragmatic hernia
Mechanical hypoventilation

Note: During their first 1–2 hr, neonatal foals may have higher P_{CO_2} values than adult horses. (Stewart JH, Rose RJ, Barko AM. 1984. Respiratory studies in foals from birth to seven days old. Equine Vet J 16:323-328).

C. Disorders and pathogeneses (see Table 9.9 and associated text)
   1. Loss of H^+ from body
      a. Gastric loss: vomiting, pyloric obstruction (functional or mechanical)
      b. Renal loss
         (1) Loop or thiazide diuretics
         (2) Secondary to respiratory acidosis
         (3) Hypokalemia
   2. Shift of H^+ from ECF to ICF due to hypokalemia
   3. Administration of sodium bicarbonate or organic anions that generate HCO_3^-
   4. Contraction alkalosis: loss of HCO_3^--poor fluid resulting in hypovolemia and increased plasma [HCO_3^-] (see Chap. 9)
D. Physiologic response
   1. In nonrenal disorders associated with metabolic alkalosis, the kidneys are expected to decrease the secretion of H^+ (exception: see paradoxical aciduria [discussed below]) and conserve less HCO_3^-.
      a. With increased plasma [HCO_3^-], the amount of HCO_3^- that enters the filtrate may exceed the capacity of the proximal tubules to conserve it.
      b. Excess HCO_3^- will be secreted by intercalated cells (Type B) of the distal nephron.
   2. Alkalemia inhibits the central respiratory chemoreceptors and thus causes hypoventilation. Hypoventilation decreases the excretion of CO_2 (thus elevates the blood P_{CO_2}) and increases the [H^+] (compensatory respiratory acidosis).
   3. "Paradoxical" aciduria
      a. When an animal has concurrent alkalosis, hypochloremia, and hypovolemia, the kidneys may produce acidic urine (see Figs. 9.3 and 9.4).
         (1) Hypovolemia stimulates the resorption of Na^+ and Cl^- in the tubules through the actions of aldosterone and angiotensin II.
         (2) Na^+ resorption is not always accompanied by Cl^- (because of Cl^- depletion) and thus an electrochemical gradient is established that promotes the secretion of H^+ (thus aciduria) and K^+, which contributes to a concurrent hypokalemia.
         (3) The secretion of H^+ by tubules also increases the generation of HCO_3^-, which adds to the severity of the metabolic alkalosis.
      b. It is seen most frequently in cattle but also may be seen in other mammals.

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b. Strong anions (Cl^-, SO_4^{2-}, lactate, acetoacetate, β-hydroxybutyrate, and other acidic 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., add Na^+ or remove lactate), then H^+ shifts into the ECF to make it 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").
3. An *alkalosis* is a condition in which there is an excess of strong cations ("bases") or a deficit of strong anions ("acids").
4. *Weak electrolytes* are those electrolytes that are in equilibria in physiologic fluids.
   a. Weak cations: NH_4^+, H^+, 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 nonvolatile weak acids ($HA + A^-$). In plasma, these occur as various anionic forms of PO_4 and albumin.
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.
7. By rearranging Eq. 10.5.a, it is shown that SID_{true} also equals the difference between the sum of weak cation concentrations and the sum of weak anion concentrations (Eq. 10.5.b), an important concept for interpreting calculated SID values.

C. Using the same concepts as Stewart,[10] Singer and Hastings[16] defined *buffer base*, which is identical to Stewart's SID.[2]

III. SID equations

A. SID_{true} (Eq. 10.6.a):[11] This equation is consistent with the SID definition but is not useful because concentrations of many analytes in the formula are not measured in clinical laboratories.

$$\begin{aligned} SID_{true} &= (\text{sum of strong cations}) - (\text{sum of strong anions}) \quad \textbf{(10.6.a.)} \\ &= ([Na^+] + [K^+] + [fCa^{2+}] + [fMg^{2+}]) - ([Cl^-] + [uSA^-]) \end{aligned}$$

uSA^- includes SO_4^{3-}, lactate, acetoacetate, β-hydroxybutyrate, other acidic anions of metabolism, and exogenous anions.

$$SID_{measured} = ([Na^+] + [K^+]) - [Cl^-] \quad \textbf{(10.6.b.)}$$

$$SID_{approximate} = [Na^+]_{mean\ normal} - [Cl^-]_{corrected} \quad \textbf{(10.6.c.)}$$

$$\text{where } [Cl^-]_{corrected} = [Cl^-]_{patient} \times \frac{[Na^+]_{mean\ normal}}{[Na^+]_{patient}}$$

and the "mean normal" concentrations of Na^+ and Cl^- are for the appropriate reference intervals for the patient's values

B. $SID_{measured}$ (Eq. 10.6.b):[15] This equation estimates the SID by using 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 uSA^-. This is probably one of the more common clinical SID formulas.

C. $SID_{approximate}$ (Eq. 10.6.c)[14]

1. The basis of the $[Cl^-]_{corrected}$ is that changes in $[Na^+]$ are expected to be matched by

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changes in $[Cl^-]$. By calculating a $[Cl^-]_{corrected}$ based on relative changes in $[Na^+]$, then one assumes that any additional change in $SID_{approximate}$ is due to changes in other anions (i.e., HCO_3^-, albumin, PO_4, or "other strong anions").

2. The equation is based on assumptions and estimations and thus is prone to be inaccurate. A major assumption is that all animals start with a mean $[Na^+]$ of a "normal" animal. Based on actual distribution of $[Na^+]$ in healthy animals, such an assumption is rarely true. There is also an assumption that a "mean normal" concentration for one laboratory will apply to other laboratories. Again, this assumption is typically not true. If the formula is used, then a "mean normal" concentration appropriate for the laboratory method (same as used for patient's $[Na^+]$) should be used.

D. Other SID formulas have been proposed that include some but not all of the strong ions.[11,17] Also, there are not standardized names for SID formulas and thus SID information should be interpreted carefully. For example, some formulas contain $[fCa^{2+}]$, $[fMg^{2+}]$, or lactate concentration but not acetoacetate or β-hydroxybutyrate concentrations. Reference intervals for estimated SID values and possible causes for abnormal estimated SID values will vary depending on the analytes that are or are not used in the formulas.

IV. Comparison of SID formulas

A. The relationship between SID_{true} and $SID_{measured}$ is shown in Eq. 10.7.

Given Eq. 10.6.a. and 10.6.b., **(10.7.)**

$$SID_{true} = SID_{measured} + ([fCa^{2+}] - [fMg^{2+}]) - [uSA^-]$$

$$\text{or } SID_{measured} = SID_{true} - [fCa^{2+}] + [fMg^{2+}]) + [uSA^-]$$

1. $SID_{measured}$ is a good approximation of SID_{true} if concentrations of fCa^{2+}, fMg^{2+}, and uSA^- are small.
2. However, when there is a high $[uSA^-]$ such as occurs in lactic acidosis or ketoacidosis, the $SID_{measured}$ value will be greater than the SID_{true}; i.e., the $SID_{measured}$ overestimates the SID_{true}.

B. It is more difficult to compare $SID_{approximate}$ with the other SID formulas. However, the $SID_{approximate}$ would also overestimate the SID_{true} if there were an increased $[uSA^-]$.

C. The changes that occur in the SID values that result from changes in weak acid concentrations can be seen by examining Equations 10.8.a–10.8.f. As shown in Eq. 10.8.e, changes in $SID_{measured}$ occur when the sum of $[HCO_3^-]$, $[Alb^{x-}]$, $[PO_4^{y-}]$, and $[uSA^-]$ changes.

Given Eq. 10.5.b. and 10.6.a.,

$$SID_{true} = ([OH^-] + [HCO_3^-] + [CO_3^{2-}] + [Alb^{x-}] + [PO_4^{y-}]) - ([H^+] + [NH_4^+]) \quad \textbf{(10.8.a.)}$$

Because typical plasma concentrations of OH^-, CO_3^{2-}, H^+, and NH_4^+ are relatively small,

$$SID_{true} \approx [HCO_3^-] + [Alb^{x-}] + [PO_4^{y-}] \quad \textbf{(10.8.b.)}$$

Given $[Alb^{x-}] + [PO_4^{y-}] \approx [A_{TOT}]$,

$$SID_{true} \approx [HCO_3^-] + [A_{TOT}] \quad \textbf{(10.8.c.)}$$

Given Eq. 10.7 and that typical plasma $[fCa^{2+}]$ and $[fMg^{2+}]$ are relatively small, **(10.8.d.)**

$$SID_{true} \approx SID_{measured} - [uSA^-]$$

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Given Eq. 10.8.b. and 10.8.d.,

$$SID_{measured} - [uSA^-] \approx [HCO_3^-] + [Alb^{x-}] + [PO_4^{y-}]$$
$$\text{or } SID_{measured} \approx [HCO_3^-] + [Alb^{x-}] + [PO_4^{y-}] + [uSA^-] \quad \textbf{(10.8.e.)}$$

In terms of A_{TOT},

$$SID_{measured} \approx [HCO_3^-] + [A_{TOT}] + [uSA^-] \quad \textbf{(10.8.f.)}$$

V. Interpretation of abnormal $SID_{measured}$ values

A. According to Stewart's method and definitions, changes in SID_{true} result in either the creation of an alkalosis or acidosis. Based on the definitions and the calculation of $SID_{measured}$, the major types of acid-base disorders are listed in Table 10.7.

B. Based on the definitions, changes in concentrations of two ions may result in a mixed acid-base disorder; e.g., concurrent hypernatremic alkalosis and hyperalbuminemic acidosis. The concurrent alkalosis and acidosis in the Stewart method raises the question of whether there is noSIDosis.

Table 10.7. Classification of acid–base disorders from P_{CO_2}, A_{TOT}, and SID

Change in independent variable		Change in measured analyte	Classification	Expected dependent variable change $[HCO_3^-]$	pH
P_{CO_2}	↓	↓ P_{CO_2}	Respiratory alkalosis	WRI	↑
	↑	↑ P_{CO_2}	Respiratory acidosis	WRI	↓
A_{TOT}	↑	↑ albumin[a]	Hyperalbuminemic acidosis	↓	↓
	↑	↑ PO_4	Hyperphosphatemic acidosis	↓	↓
	↓	↓ albumin[b]	Hypoalbuminemic alkalosis	↑	↑
SID_t / SID_m[c]	↑ / ↑	↑ Na^+	Hypernatremic or contraction alkalosis[d]	↑	↑
	↑ / ↑	↓ Cl^-	Hypochloremic alkalosis	↑	↑
	↓ / ↓	↓ Na^+	Hyponatremic or dilutional acidosis[e]	↓	↓
	↓ / ↓	↑ Cl^-	Hyperchloremic acidosis	↓	↓
	↓ / WRI	None[f]	Metabolic acidosis and ↑ $[uSA^-]$[g]	↓	↓
	↓ / ↑	Variable[h]	Metabolic acidosis and ↑ $[uSA^-]$[i]	↓	↓

[a] If hyperproteinemia, then hyperproteinemic acidosis.

[b] If hypoproteinemia, then hypoproteinemic alkalosis.

[c] $SID_t = SID_{true}$; $SID_m = SID_{measured}$.

[d] With loss of free water, $[Na^+]$ and $[Cl^-]$ increase proportionately but $[Na^+]$ increases more than $[Cl^-]$ on an absolute basis. Therefore, SID increases (but mildly).

[e] Opposite explanation of hypernatremic alkalosis.

[f] Concentrations of Na^+, K^+, and Cl^- are all WRI.

[g] Because the $SID_{measured}$ is WRI, the acidosis would be recognized only if it is known that either a ↓ $[HCO_3^-]$ or an ↑ anion gap (which indicates ↑ $[uSA^-]$) is present.

[h] Could be found in hyponatremia, normonatremic, or hypernatremic animals; the $SID_{measured}$ is increased because the difference between $[Na^+ + K^+]$ and $[Cl^-]$ is increased.

[i] The acidosis would be recognized if it is known that either a ↓ $[HCO_3^-]$ or an ↑ anion gap (which indicates ↑ $[uSA^-]$) is present.

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Table 10.8. Diseases and conditions that cause hypoxemia

Decreased inhaled O_2 content: high altitude, closed ventilation area
Impaired respiratory exchange: respiratory obstruction, hypoventilation
Decreased alveolar function: pneumonia, emphysema, pulmonary ventilation-perfusion imbalance, right-to-left shunt, congestive heart failure, neonatal respiratory distress syndrome

Note: Compared to adult horses, neonatal foals have lower Po_2, higher Pco_2, higher HCO_3^-, and slightly lower pH values because of underdeveloped lungs.

VI. As recommended by some authors, SID values should be interpreted with routine blood gas values (pH, Pco_2, $[HCO_3^-]$) to determine if the animal has an acid-base problem and if it is respiratory or nonrespiratory (metabolic).[13,14] If it 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 electrolytes disorder was obtained through the interpretation of pH, Pco_2, $[HCO_3^-]$, BE_B, $[Na^+]$, $[K^+]$, $[Cl^-]$, anion gap, and albumin concentration (see Acid-Base Abnormalities section above and Chap. 9). Evaluation of such information has and will provide an understanding of an animal's pathologic state.

HYPOXEMIA

I. Hypoxia (decreased oxygen delivery or utilization of O_2 by tissues) may be due to hypoxemia (decreased dissolved O_2 in blood; decreased Po_2) or other causes.
   A. Hypoxia due to hypoxemia (Table 10.8).
      1. *Atmospheric hypoxia:* inhalation of atmosphere that has decreased O_2 content (e.g., high altitude, anesthetic problem, in an air-tight box)
      2. *Tidal hypoxia:* decreased O_2 uptake because of impaired respiratory exchange (e.g., respiratory obstruction, hypoventilation due to anesthetic agents)
      3. *Alveolar hypoxia:* decreased O_2 uptake due to decreased alveolar function (e.g., pneumonia, emphysema, pleural effusion, or intrapulmonary exudates)
   B. Other causes of hypoxia include the following:[18]
      1. *Hemoglobic hypoxia:* decreased O_2 bound to Hgb (e.g., methemoglobinemia, carbon monoxide poisoning or decreased Hgb (e.g., anemia). (Note: There will be decreased blood oxygen content but the blood P_aO_2 will not be decreased.)
      2. *Stagnant hypoxia:* increased O_2 use by tissue because of poor blood circulation (e.g., shock, blood vessel occlusion, hyperviscosity syndrome)
      3. *Histotoxic hypoxia:* defective O_2 use by tissues because of interference with metabolic pathways (e.g., some drugs, alcohol)
      4. *Demand hypoxia:* increased O_2 demand by hyperfunctioning cells (e.g., hyperthyroidism, pyrexia)

II. Expected Po_2
   A. Arterial blood is the only acceptable sample for the assessment of oxygenation of blood by the respiratory system. The expected P_aO_2 varies with the FiO_2, the O_2 content of the inhaled gas. (FiO_2 in decimal fraction, P_aO_2 in mmHg): (0.2, 95–100), (0.3, 150), (0.4, 200), (0.5, 250), (0.8, 400), (1.0, 500).[19]

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B. For room air, the FiO_2 is near 0.2 and thus a P_aO_2 of 90–100 mmHg is expected. Some authors consider a $P_aO_2 < 80$ mmHg to be hypoxemia.[19] Hypoxemic stimulus of chemoreceptors is reported to occur when $P_aO_2 < 70$ mmHg[20] or < 60 mmHg.[21]

C. With the increased use of point-of-care instruments that have O_2 electrodes, more P_vO_2 values are being measured. In free-flowing venous blood in an animal with a P_aO_2 near 95 mmHg, the P_vO_2 will be near 40 mmHg. If the sample is collected from an occluded vein, the P_vO_2 will be lower. If collected from capillary blood, the PO_2 should be between the P_aO_2 and P_vO_2 values.

D. Compared to the adult horse, the foal at birth has lower P_aO_2 (< 40 mmHg) and higher P_aCO_2 values (> 50 mmHg) during its first few hours. The P_aO_2 and P_aCO_2 slowly change to adult values by 4–7 days of age.[22]

E. PO_2 is a measure of the tension created by dissolved O_2 in the plasma and is not dependent on blood [Hgb]. With anemia, there will be less O_2 in blood (blood O_2 content decreased) and there may be less O_2 delivered to tissues (thus hypoxia may be present), but the PO_2 will be WRI as long as lungs can properly oxygenate the blood.

III. SpO_2

A. A decreasing SpO_2 typically indicates the development of hypoxemia but only when the P_aO_2 is less than 90 mmHg. With greater FiO_2 such as occurs during anesthesia, greater P_aO_2 values are expected. Because of the shape of the oxyhemoglobin dissociation curve, falling S_aO_2 values may not be detected until the P_aO_2 is less than 90 mmHg, which might be considerably less than expected for high FiO_2 values.

B. Erroneous values[3]

1. With increasing concentrations of COHgb (as in carbon monoxide poisoning), the SpO_2 remained above 90% even when O_2Hgb percentage decreased to 30% because the oximeter perceived COHgb to be O_2Hgb. As shown in Eq. 10.4.b, the O_2Hgb percentage will decrease with increased concentrations of COHgb or MetHgb.
2. Similar but less severe interference was seen with increasing concentrations of MetHgb.

C. In horses, the SpO_2 underestimated the S_aO_2 when $S_aO_2 \geq 90\%$ by an average of 4.4% and overestimated the S_aO_2 when $S_aO_2 \leq 90\%$ by an average of 4.1%.[23] The errors might be due to different properties of equine blood compared to human blood.

D. In dogs with $S_aO_2 \geq 70\%$, the average difference between SpO_2 and S_aO_2 was about 3%–4% (e.g., SpO_2 of 87% and S_aO_2 of 90%), depending on application site (tongue or tail) and type of probe (ear or finger). The reason for the difference is not known.[4]

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Chapter 11

CALCIUM, PHOSPHORUS, MAGNESIUM, AND THEIR REGULATORY HORMONES

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Table 11.1. Abbreviations and symbols in Chapter 11

»	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
1,25-DHCC	1,25-dihydroxycholecalciferol (calcitriol)
24,25-DHCC	24,25-dihydroxycholecalciferol
25-HCC	25-hydroxycholecalciferol (calcidiol)
ATPase	adenosine triphosphatase
Ca^{2+}	calcium
ECF	extracellular fluid
fCa^{2+}	free ionized calcium
fMg^{2+}	free ionized magnesium
GFR	glomerular filtration rate
GH	growth hormone
HHM	humoral hypercalcemia of malignancy
ICF	intracellular fluid
Mg^{2+}	magnesium
Pi	inorganic phosphorus
PO_4	inorganic phosphate (all forms)
PTH	parathyroid hormone
PTHrp	parathyroid hormone-related protein
SI	Système International d'Unités
tCa^{2+}	total calcium
tMg^{2+}	total magnesium
URL	upper reference limit
WRI	within reference interval

TOTAL CALCIUM (tCa^{2+}) CONCENTRATION

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 bound to anionic molecules.
      1. fCa^{2+}: about 50% of [tCa^{2+}]; present as free ions in plasma H_2O; the portion of tCa^{2+} that is hormonally regulated and contributes to pathologic states
      2. Anion-bound Ca^{2+}
         a. Bound to anionic proteins: About 40%–45% of tCa^{2+} is bound to negatively charged sites on proteins (4 times more to albumin than 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.
         b. Bound to nonprotein anions: About 5%–10% of tCa^{2+} is bound to citrates, PO_4, lactate, and other small, diffusable anions.
   B. Major factors that determine serum [tCa^{2+}] (Fig. 11.1)
      1. Age: Young dogs (6–24 weeks old) have serum [tCa^{2+}] about 1–2 mg/dL greater than mature dogs.[2] Young foals (1–60 days) have serum [tCa^{2+}] similar to adult values.[3] Kittens (4–6 weeks to 20–24 weeks old) have serum [tCa^{2+}] similar to adult values.[4] Age-related data were not found for calves.
      2. Because serum Ca^{2+} concentrations in common chemistry profiles represent [tCa^{2+}], a

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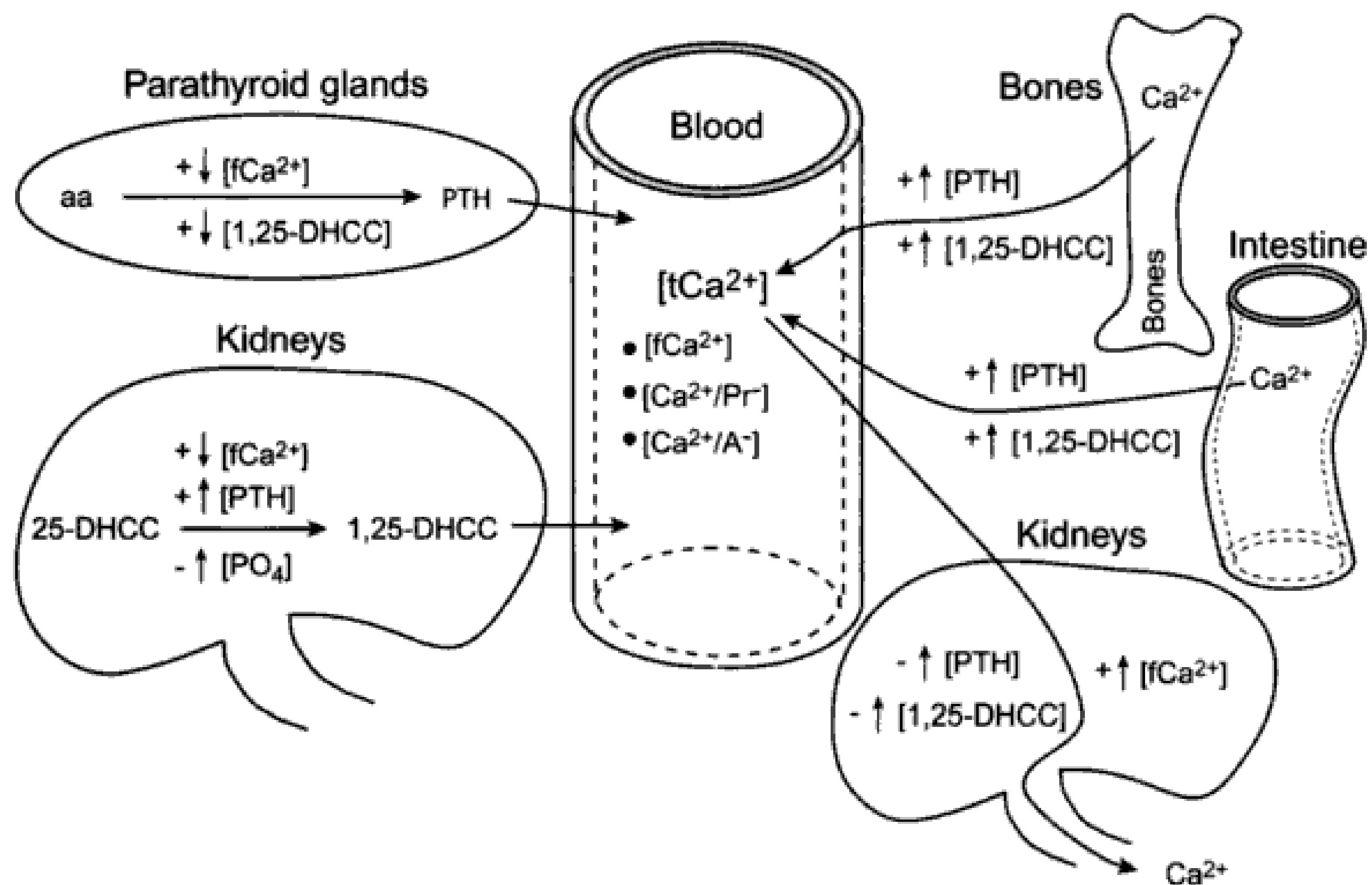

Fig. 11.1. Relationships of calcium kinetics and the production of PTH and 1,25-DHCC. (+ is positive effector [stimulates the process]; - is negative effector [inhibits the process]; Ca^{2+}/Pr^- is calcium bound to protein; Ca^{2+}/A^- is calcium bound to nonprotein anions.)

- Ca^{2+} is present in plasma in three forms: fCa^{2+}, Ca^{2+} bound to proteins, and Ca^{2+} bound to small anions such as citrate and PO_4.
- PTH production in parathyroid glands is stimulated by ↓ [fCa^{2+}] and ↓ [1,25-DHCC] and inhibited by ↑ [fCa^{2+}] and ↑ [1,25-DHCC].
- Conversion of 25-HCC to 1,25-DHCC in kidneys is catalyzed by 1α-hydroxylase. The activity of 1α-hydroxylase is promoted by ↓ [fCa^{2+}] and ↑ [PTH] and inhibited by ↑ [fCa^{2+}] and ↑ [PO_4].
- Ca^{2+} mobilization from bone and Ca^{2+} absorption in intestine are promoted by ↑ [1,25-DHCC] and ↑ [PTH] (less Ca^{2+} mobilization and absorption occurs if there is ↓ [1,25-DHCC] or ↓ [PTH]).
- Urinary excretion of Ca^{2+} is enhanced by ↑ [fCa^{2+}] and excretion is reduced by ↓ [fCa^{2+}], ↑ [PTH], and ↑ 1,25-DHCC (increased excretion of anions may obligate Ca^{2+} excretion and thus decreases [fCa^{2+}]).

(Note: Horse kidneys lack 1α-hydroxylase and thus do not form 1,25-DHCC.)

decrease in serum protein concentration (especially hypoalbuminemia) causes a decrease in the bound Ca^{2+} and thus may cause hypocalcemia.

3. Absorption of Ca^{2+} in intestine (mostly in ileum, but from duodenum to colon)
   a. In dogs, cats, and cattle, intestinal Ca^{2+} absorption requires vitamin D to induce mucosal epithelial cell synthesis of Ca^{2+}-binding proteins. PTH activity augments vitamin D actions to increase Ca^{2+} absorption, mostly by stimulating 1,25-DHCC production.
   b. Based on renal failure studies in horses, Ca^{2+} absorption in the equine intestine is not as dependent on vitamin D and is more dependent on the amount of dietary Ca^{2+}.[5] Equine kidneys lack 1α-hydroxylase (thus do not convert 25-HCC to 1,25-DHCC) but can produce small quantities of 24,25-DHCC.[6]
4. Resorption from or deposition of Ca^{2+} in bone[7]

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a. PTH stimulates Ca^{2+}-pumps in the osteocyte membrane system that promote movement of Ca^{2+} from bone to bone fluid to ECF. Secondarily, PTH induces osteoblasts to change shape and allow osteoclasts to contact bone matrix, or to release substances that stimulate osteoclasts to degrade bone by enzymatic digestion and acidification.[8]
b. Vitamin D either promotes osteoclastic activity or permits osteolytic cells to respond to PTH activity.
c. Calcitonin blocks osteoclastic osteolysis through direct changes in osteoclasts and by reducing activation of osteoprogenitor cells. However, with continuous PTH stimulation, osteoclasts escape from the suppressive effects of calcitonin.

5. Resorption of fCa^{2+} from tubular fluid in kidney tubules[9]
a. Ca^{2+} (free and bound to small anions) passes freely through the glomerular filtration barrier; protein-bound Ca^{2+} should not pass through. About 66% of filtered Ca^{2+} is resorbed passively in proximal tubules with a Na^{+}-Ca^{2+} cotransport system, 25% in the ascending limb of the loop of Henle, and the remainder is resorbed in the distal tubules. PTH regulates only the distal fraction by activating a hormone-specific adenylate cyclase system. Vitamin D plays a relatively minor role by promoting Ca^{2+} resorption through the formation of calbindin, a Ca^{2+}-binding protein in the distal nephron.
b. Angiotensin II stimulates the resorption of Na^{+} in the proximal tubules via a Na^{+}-Ca^{2+} cotransport system; Ca^{2+} is concurrently resorbed.

C. Ca^{2+} and PO_4 interaction
1. [fCa^{2+}] and [PO_4] in plasma are great enough in healthy animals that $Ca_3(PO_4)_2$ complexes would form if there were not inhibitors present.[10]
2. $Ca^{2+} \times PO_4$: When the product of [tCa^{2+}] and [Pi] (both in mg/dL) exceeds 70, metastatic mineralization of tissues (kidneys, lungs) tends to occur.

II. Analytical concepts

A. Sample
1. Serum is the preferred sample; heparinized plasma may be used in some assays.
2. Blood anticoagulants that bind Ca^{2+} (EDTA, citrate, oxalate) should not be used in samples for Ca^{2+} assays.
3. In people, prolonged venous occlusion during blood collection may increase [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. In some systems, aerobic sample handling may increase measured [tCa^{2+}] by 0.4 mg/dL when the sample's pH increases because of the loss of CO_2.

C. Unit conversion: mg/dL $\times$ 0.2495 = mEq/L $\times$ 0.5 = mmol/L (SI unit, nearest 0.02 mmol/L)[11]

D. Because a large portion of tCa^{2+} is protein-bound but the body regulates [fCa^{2+}], [tCa^{2+}] may be decreased because of hypoproteinemia or hypoalbuminemia when the animal does not have a defect in regulating [fCa^{2+}]. To estimate the effect of lower protein and albumin concentrations, correction or adjusting formulas have been proposed.
1. Canine adjusted [tCa^{2+}] considering albumin concentration (Eq. 11.1.a)[2]

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$$\text{Canine adjusted } [tCa^{2+}] = \text{measured } [tCa^{2+}] - \text{measured [albumin]} + 3.5\ (\pm 1.3) \qquad \textbf{(11.1.a.)}$$

Example: If $[tCa^{2+}] = 8.0$ mg/dL & $[\text{albumin}] = 1.0$ g/dL;
Canine adjusted $[tCa^{2+}] = 8.0 - 1.0 + 3.5\ (\pm 1.3) = 10.5 \pm 1.3 = 9.2$ to 11.8 mg/dL
Interpretation: If the dog was not hypoalbuminemic, its serum $[tCa^{2+}]$ would be from 9.2 to 11.8 mg/dL in 95% of canine samples.

$$\text{Canine adjusted } [tCa^{2+}] = \text{measured } [tCa^{2+}] - (0.4 \times \text{measured [TP]}) + 3.3\ (\pm 1.6) \qquad \textbf{(11.1.b.)}$$

Example: If $[tCa^{2+}] = 8.0$ mg/dL & $[TP] = 4.0$ g/dL;
Canine adjusted $[tCa^{2+}] = 8.0 - (0.4 \times 4.0) + 3.3\ (\pm 1.6) = 9.7 \pm 1.6 = 8.1$ to 11.3 mg/dL
Interpretation: If the dog was not hypoproteinemic, its serum $[tCa^{2+}]$ would be from 8.1 to 11.3 mg / dL in 95% of canine samples.

a. Some authors do not include the "± 1.3," which is an estimate of the 95% confidence interval of the published data.
b. The formula should be used cautiously for three reasons: (a) the adjusted $[tCa^{2+}]$ is at best an estimate; (b) 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; (c) 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:globulin ratios; e.g., samples with panhypoproteinemia versus those with hypoalbuminemia and hyperglobulinemia.

2. Canine adjusted $[tCa^{2+}]$ considering total protein concentration (Eq. 11.1.b.)
   a. Some authors do not include the "± 1.6," which is an estimate of the 95% confidence interval of the published data.
   b. Even though the formula does include total protein concentration, it should be used cautiously for reasons mentioned above.
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.[12,13] Although the general principle that $[tCa^{2+}]$ varies with [albumin] is true, the weakness and variation in the relationship among individuals suggests that rigid correction formulas may not be appropriate, especially in cattle.
4. It is recommended that the potential effects of dysproteinemia be considered when interpreting $[tCa^{2+}]$. If the formulas for adjusting the measured concentrations in canine sera are used, the calculated values should be considered only rough approximations.

III. Hypercalcemia (Table 11.2)

A. Increased Ca^{2+} mobilization from bone or absorption in intestine

1. Increased [PTH] or [PTHrp]
   a. Primary hyperparathyroidism[14-16]
      (1) Parathyroid adenomas or carcinomas secrete PTH that stimulates Ca^{2+} resorption from bone and increases Ca^{2+} absorption in the intestine. Renal excretion of Ca^{2+} may be increased because hypercalcemia causes an increased filtered load (more filtered than can be resorbed even with increased PTH activity) but not enough to prevent hypercalcemia.
      (2) Hypophosphatemia is expected because PTH is a potent phosphaturic agent. If there is a decreased GFR, there may be normophosphatemia or hyperphos-

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ed with malignancies in bone. The hypercalcemic agents may be working locally or systemically and may include interleukin-1, tumor growth factor, and tumor necrosis factor.[18]

b. [Pi] may be within reference intervals or increased.

B. Decreased urinary excretion of Ca^{2+}
1. Renal insufficiency or failure
a. Horses with some acute or chronic renal diseases
(1) In health, equine kidneys excrete excess dietary Ca^{2+}. Renal disease that causes decreased GFR results in impaired renal excretion of Ca^{2+} and thus hypercalcemia.[41] Lowering dietary Ca^{2+} intake by switching from alfalfa hay to grass hay can reduce or eliminate the hypercalcemia but the impaired GFR persists.[42] Alfalfa hay can contain 2–10 times the Ca^{2+} content of grass or mixed hay.[43]
(2) Hypophosphatemia may be present.
(3) Horses with hypercalcemic renal failure have decreased [PTH].[44]
b. Dogs and cats with acute or chronic renal disease
(1) Occasional dogs and cats with acute renal failure are hypercalcemic. The hypercalcemia may be due to increased concentration of Ca^{2+} bound to citrate or PO_4.
(2) 10%–15% of dogs with chronic renal failure are reported to be hypercalcemic, which may be due to binding of Ca^{2+} to retained anions. However, most dogs with chronic renal failure have low-normal to mildly decreased [tCa^{2+}].
2. Canine hypoadrenocorticism (Addison's disease)
a. About 30% of dogs with hypoadrenocorticism are hypercalcemic.[7,45]
b. Adrenalectomized dogs decrease Ca^{2+} excretion by excessive tubular resorption of Ca^{2+}.[46] The reason for enhanced tubular resorption is not established but may involve angiotensin II. When the dog with hypoadrenocorticism becomes hypovolemic due to vomiting, diarrhea, or impaired renal concentrating ability, angiotensin II activity increases. Angiotensin II promotes Na^+ resorption in proximal renal tubules via a Na^+-Ca^{2+} cotransport system.[9] Thus, enhanced resorption of Na^+ may also promote the proximal tubular resorption of Ca^{2+} and cause hypercalcemia. Concurrent hemoconcentration itself may slightly increase serum [tCa^{2+}].
3. Thiazide diuretics: Thiazide diuretics act in the distal nephron to promote hypernatruria and, secondarily, volume depletion. Hypovolemia promotes enhanced proximal tubular resorption of Na^+ and, secondarily, proximal tubular resorption of Ca^{2+}.[9] This form of hypercalcemia is rarely reported in domestic animals but does occur in dogs.[47]

C. Increased protein-bound Ca^{2+}: In some cases of marked hyperproteinemia associated with multiple myeloma, there is an increase in negatively charged globulins that bind cations including Ca^{2+}. 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 increased [tCa^{2+}] and [fCa^{2+}] WRI.

D. Other or unknown mechanisms
1. Intravenous infusion of Ca^{2+}: if the rate of administration exceeds the rate of renal excretion
2. Hemoconcentration: If hypercalcemia occurs, expect it to be a mild hypercalcemia and

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associated with increased protein-bound or small anion-bound Ca^{2+}. It may also be related to angiotensin II–stimulated transport of Na^+ and Ca^{2+} in proximal tubules.

3. Juvenile-onset hypothyroidism:[48] may be due to increased intestinal absorption and decreased renal excretion of Ca^{2+}
4. Retained fetus and endometritis in a dog[49]

IV. Hypocalcemia (Table 11.3)

A. Hypoalbuminemic hypocalcemia (hypoproteinemic hypocalcemia)[2]

1. Hypocalcemia results from a decreased concentration of negatively charged proteins and therefore of protein-bound Ca^{2+}; regulation of [fCa^{2+}] would be adequate in these animals unless there is a concurrent defect in regulation of [fCa^{2+}].
2. It has been called pseudohypocalcemia because there is not a decrease in [fCa^{2+}] and clinical signs of hypocalcemia do not occur. However, the animal truly has hypocalcemia when the [tCa^{2+}] is lower than the appropriate reference interval.

Table 11.3. Diseases and conditions that cause hypocalcemia

- [a]Hypoalbuminemia (hypoproteinemia)
- Decreased PTH concentration or activity
  - Primary hypoparathyroidism (damaged parathyroid gland)
  - Pseudohypoparathyroidism (decreased PTH receptor responsiveness)
  - Hypomagnesemia (bovine grass tetany)
- Inadequate Ca^{2+} mobilization from bone or absorption in intestine
  - Hypovitaminosis D
    - [a]Chronic renal disease/failure in dogs, cats, and cattle
    - Dietary vitamin D deficiency (rare)
  - Exocrine pancreatic insufficiency (dogs)
  - [a]Pregnancy, parturient, or lactational hypocalcemia (milk fever, puerperal tetany)
  - Hypercalcitonism: thyroid C-cell neoplasia, iatrogenic (calcitonin therapy)
  - Nutritional hypocalcemia (rare)
  - Oxalate toxicity
- Excess urinary excretion of Ca^{2+}
  - Ethylene glycol toxicosis (dogs and cats)
  - Intravenous HCO_3^- infusions
  - Furosemide treatment
- Ca^{2+} binding with diffusable anions
  - Ca^{2+}-binding anticoagulants: EDTA, citrate, oxalate (*in vivo* or *in vitro*)
  - Tetracycline administration
- Other or unknown mechanisms
  - [a]Acute pancreatitis in dogs
  - Urinary tract obstruction
  - Acute renal failure
  - Phosphate enemas in cats
  - Blister beetle poisoning (cantharidiasis) in horses
  - Myopathies: transport tetany, exertional rhabdomyolysis, malignant hypothermia, endurance-type exercise
  - Acute tumor lysis syndrome
  - Rumen overload (acute carbohydrate ruminal engorgement)

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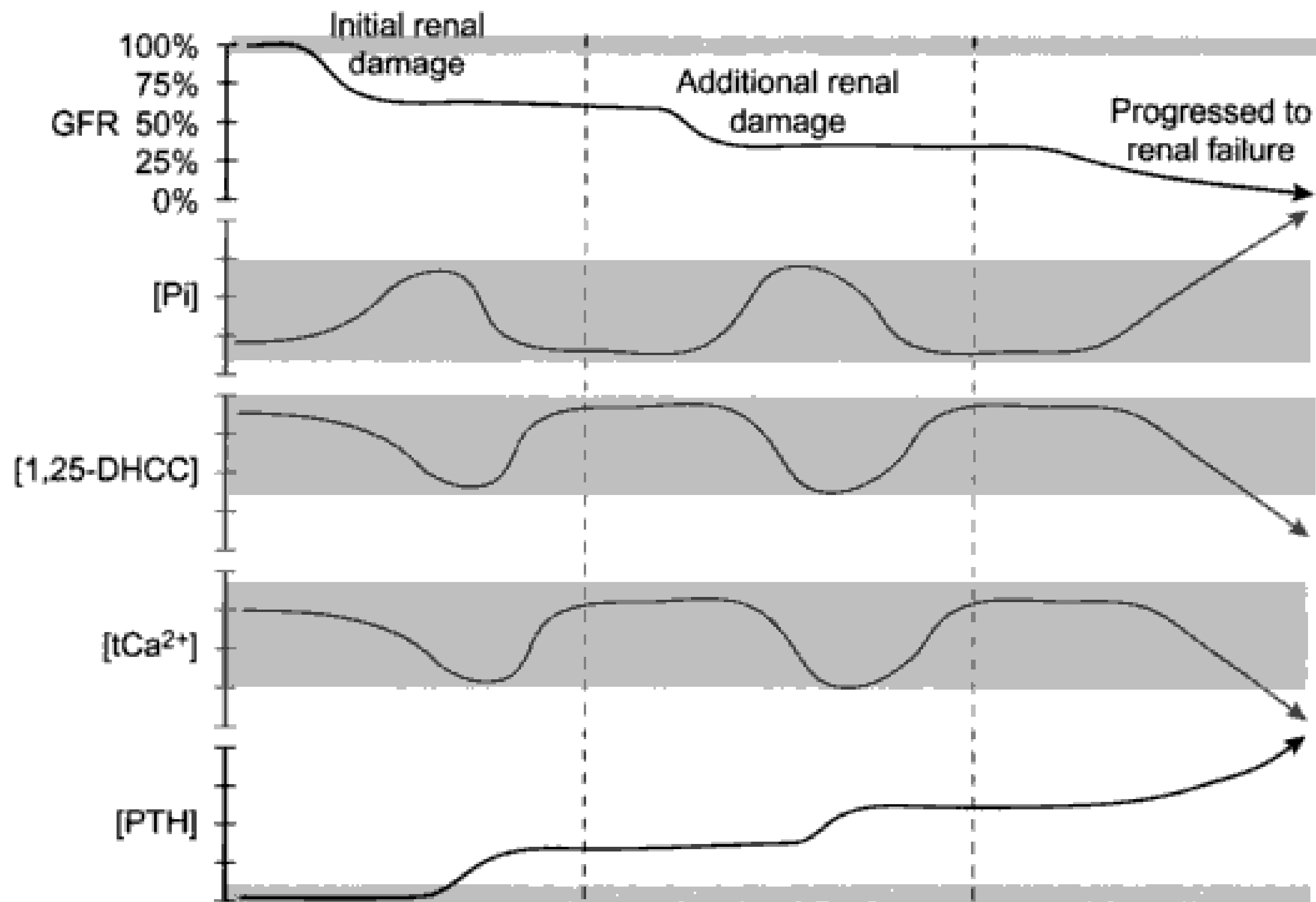

Fig. 11.3. Schematic pathogenesis of secondary renal hyperparathyroidism in dogs, cats, and cattle. Shaded areas represent reference intervals for GFR or each analyte concentration.

- *Initial renal damage:* The sequence of events described in Fig. 11.2 occurs to compensate for the decreased clearance of PO_4 and inadequate activation of vitamin D. A new homeostasis in Ca^{2+} and PO_4 balance is maintained by ↑ PTH secretion.
- *Additional renal damage:* As renal disease progresses and more nephrons are lost, pathophysiologic responses recur which stimulate more PTH synthesis to attempt to maintain physiologic concentrations of fCa^{2+}, PO_4, and 1,25-DHCC.
- *Progressed to renal failure:* Eventually, renal disease causes sufficient reduction in GFR so that serum [Pi] does not return to normal and insufficient 1,25-DHCC 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.

(1) When seen, often associated with peak lactation and corresponding peak loss of Ca^{2+} in milk

(2) May have concurrent hypomagnesemia and hypophosphatemia or hyperphosphatemia

c. Preparturient hypocalcemia in queens[55]

(1) Clinical signs (anorexia, depression, lethargy) occurred 3–17 days prior to parturition.

(2) Three queens had decreased [tCa^{2+}]; [fCa^{2+}] was decreased in the one cat in which it was measured.

4. Hypercalcitonism

a. Hypercalcitonism is usually due to thyroid C-cell neoplasms that are uncommon; reported most commonly in older bulls with medullary thyroid neoplasms.[56] When associated with C-cell neoplasms, there is usually only mild hypocalcemia because:

(1) Neoplasms are seen in older animals and their bones are relatively refractory to calcitonin (calcitonin does not inhibit PTH-stimulated osteoclastic activity).

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(2) Lowering of [fCa^{2+}] causes a compensatory increase in PTH production that attempts to maintain physiologic [fCa^{2+}].
(3) Marked hypocalcemia may occur if a C-cell neoplasm destroys parathyroid glands, causing hypercalcitonism and concurrent primary hypoparathyroidism.

b. Excess administration of salmon calcitonin (used to lower [tCa^{2+}] in hypervitaminosis D or HHM) could cause hypocalcemia.

5. Nutritional hypocalcemia (nutritional secondary hyperparathyroidism)
   a. Occurs with vitamin D–deficient diets and when diets are not balanced for Ca^{2+} and PO_4; i.e., dietary Ca^{2+}:PO_4 ratio is lower than the desired ratio for the species. The diet causes the hypocalcemia; hypocalcemia stimulates parathyroid gland hyperplasia. Imbalanced diets may include excessive PO_4 (meat diet for carnivores) and/or a relative or absolute deficiency of Ca^{2+}. The net effect is too much PO_4 in the body and too little Ca^{2+}.
   b. When decreased [fCa^{2+}] is present, it stimulates parathyroid glands to cause hyperparathyroidism. The increased PTH release tends to keep [fCa^{2+}] near normal at the expense of Ca^{2+} content of bones, and thus osteomalacia may be present, especially in young animals (bones of older animals are relatively refractory to PTH-stimulated osteoclastic activity).
   c. Degree of hypocalcemia depends on severity of dietary imbalance and availability of Ca^{2+} from bones (more available in young animals initially).
   d. 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 measured, expect increased 24-hr PO_4 clearance and increased fractional excretion of PO_4.
6. Oxalate toxicity[57]
   a. Horses that eat plants that have high oxalate content but low Ca^{2+} content (fuffel, pangola, *Setaria*, kikuyu, rhubarb, *Halogeton*, greasewood, soursob) absorb less dietary Ca^{2+} and develop hypocalcemia.
   b. In ruminants, *Halogeton* and curly dock (*Rumex crispus*) may cause hypocalcemia.[58]
   c. Alfalfa has high oxalate content but ingestion does not cause hypocalcemia because alfalfa also has high Ca^{2+} content.

D. Excess urinary excretion of Ca^{2+}
1. Ethylene glycol toxicosis
   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 result in intravascular formation of calcium oxalate crystals.[59]
   b. Acute nephrosis of the disorder may decrease tubular resorption of Ca^{2+}.[60,61]
2. Intravenous HCO_3^- infusions in cats
   a. Both [tCa^{2+}] and [fCa^{2+}] are reported to be decreased in some cats receiving intravenous HCO_3^-.[62]
   b. Pathogenesis of the hypocalcemia was not explained; 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+}], or 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 resorption of Ca^{2+} is dependent on gradients established by Na^+ resorption.[9,63]

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pH is abnormal. If so, results should be reported as normalized or adjusted [fCa^{2+}]. If a patient is either acidemic or alkalemic, a normalized or adjusted [fCa^{2+}] may be misleading because it does not represent the true [fCa^{2+}] in the sample.

5. Anticoagulants for whole blood and plasma samples
   a. Heparin is the only acceptable anticoagulant for measuring [fCa^{2+}] in blood or plasma samples, but heparin (a polyanion) does bind Ca^{2+}. To minimize preanalytical error, special calcium-titrated heparin tubes are recommended. If the special tubes are not available, then the heparin concentration should not exceed 15 U/mL of blood.[81]
      (1) The [fCa^{2+}] may decrease from 5%–50% by varying the amount of sodium heparinate (commonly called heparin) in the sample.[82]
      (2) 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. However, the measured [fCa^{2+}] represents Ca^{2+} from the calcium-titrated heparin and Ca^{2+} in the patient's plasma and thus appropriate reference intervals must be available.
      (3) Zinc heparinate was attempted as an anticoagulant, but the zinc interfered with the fCa^{2+} assay.[83]
   b. Other anticoagulants that bind Ca^{2+} (EDTA, citrate, oxalate) cannot be used in samples for fCa^{2+} or tCa^{2+} assays.
6. It is recommended that [fCa^{2+}] be reported with special information: type of sample, site of collection, measured [fCa^{2+}], measured pH, and [fCa^{2+}] converted to a sample pH of 7.4.[81]

B. Common clinical assays
   1. Special instruments measure [fCa^{2+}] using Ca^{2+} ion-selective electrodes via potentiometry. If the instrument also contains a pH electrode, it may measure pH and adjust the measured [fCa^{2+}] to a calculated [fCa^{2+}] that would be expected if the pH of the sample was 7.4.
   2. Different sources of standard solutions may result in different measured [fCa^{2+}].

C. Unit conversion: mg/dL × 0.2495 = mEq/L × 0.5 = mmol/L (SI unit, nearest 0.01 mmol/L)[11]

III. Abnormal concentrations
   A. Disorders of tCa^{2+} homeostasis are typically disorders of fCa^{2+} and clinical signs or pathologic events reflect abnormal [fCa^{2+}].
   B. Exceptions to the general statement involve disorders that change the bound-Ca^{2+} concentrations and not the [fCa^{2+}].
      1. Hypocalcemia due to hypoproteinemia or hypoalbuminemia
      2. Hypercalcemia in renal failure or multiple myeloma (Ca^{2+} bound to citrate, PO_4, or abnormal globulins)
      3. In urinary tract obstruction in cats, decreases in [fCa^{2+}] may be greater and more common than decreases in [tCa^{2+}]; Ca^{2+} may be bound to PO_4 or other anions not excreted by the urinary system.[67]
      4. Hyperthyroid cats may have a decreased [fCa^{2+}] but [tCa^{2+}] WRI. The pathogenesis of the free hypocalcemia is not understood. The cats do have secondary hyperparathyroidism.[84]
      5. Horses after a cross-country race had decreased [fCa^{2+}] but [tCa^{2+}] WRI. The lower

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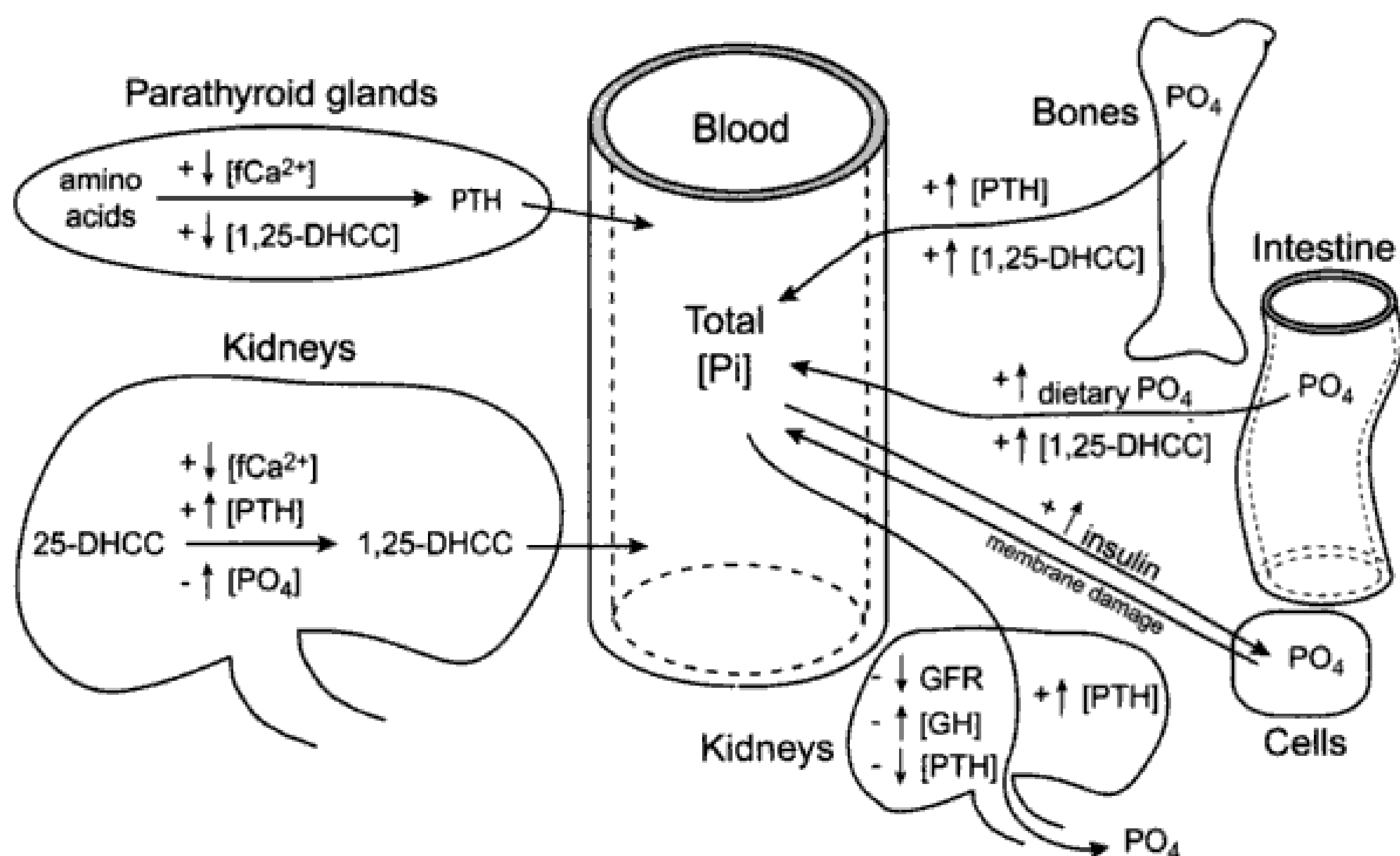

Fig. 11.4. Relationships of PO_4 kinetics and the production of PTH and 1,25-DHCC. (+ is positive effector [stimulates the process]; - is negative effector [inhibits the process].)

- PTH production in parathyroid glands is stimulated by ↓ [fCa^{2+}] and ↓ [1,25-DHCC] and inhibited by ↑ [fCa^{2+}] and ↑ [1,25-DHCC].
- Conversion of 25-HCC to 1,25-DHCC in kidneys is catalyzed by 1α-hydroxylase. The activity of 1α-hydroxylase is promoted by ↓ [fCa^{2+}] and ↑ [PTH] and inhibited by ↑ [fCa^{2+}] and ↑ [PO_4].
- PO_4 mobilization from bone is promoted by ↑ [1,25-DHCC] and ↑ [PTH]; less PO_4 mobilization occurs with ↓ [1,25-DHCC] and ↓ [PTH].
- PO_4 absorption in intestine is promoted by ↑ [1,25-DHCC] and ↑ dietary PO_4; less PO_4 absorption occurs with ↓ [1,25-DHCC] and ↓ dietary PO_4.
- Urinary excretion of PO_4 is enhanced by ↑ [PTH] and excretion is reduced by ↓ GFR, ↓ [PTH], and ↑ [GH].
- Insulin promotes the uptake of PO_4 by cells; however, cell damage will allow PO_4 to escape from the cells and enter plasma.

(*Note:* Horse kidneys lack 1α-hydroxylase and thus do not form 1,25-DHCC.)

[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.

INORGANIC PHOSPHORUS (Pi) CONCENTRATION

I. Physiologic processes
   A. Pi exists in different forms depending on pH: $H_3PO_4 \leftrightarrow H^+ + H_2PO_4^- \leftrightarrow H^+ + HPO_4^{2-} \leftrightarrow H^+ + PO_4^{3-}$. 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 will be designated as PO_4. About 10% of Pi is bound to cationic proteins, 35% is bound to nonprotein cations, and 55% is free.[1]
   B. Major factors that determine serum [Pi] (Fig. 11.4)
      1. Renal clearance of PO_4

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Table 11.4. Diseases and conditions that cause hyperphosphatemia

Decreased urinary PO_4 excretion
- *Decreased GFR
- Urinary bladder rupture or urine leakage into tissues
- Decreased [PTH] or activity (hypoparathyroidism)
- Acromegaly

Increased PO_4 absorption from intestine
- Phosphate enema
- Increased vitamin D (see Table 11.2)
- Ischemic intestinal lesions (maybe also shift from ICF to ECF)
- Diet with 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
- Osteolytic bone lesions

Note: [Pi] in growing mammals may be up to 3 mg/dL greater than [Pi] reference intervals for adults of the species. *In vitro* hemolysis or delayed removal of serum or plasma from blood sample will allow PO_4 from erythrocytes to increase the [Pi] in the serum or plasma.

D. 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.
2. Expressing [PO_4] as a [Pi] can be confusing but should not be a clinical problem if appropriate reference intervals are provided. The basis of the conversion is as follows:
   a. 1 mmol of $H_2PO_4^-$ weighs 97 mg; in 1 mmol of $H_2PO_4^-$ there are 31 mg of P; therefore 97 mg $H_2PO_4^-$/dL yields 31 mg P/dL.
   b. 1 mmol of HPO_4^- weighs 96 mg; in 1 mmole of HPO_4^- there are 31 mg of P; therefore 96 mg HPO_4^-/dL yields 31 mg P/dL. Because of the equilibrium between the different PO_4 molecules at a pH of 7.4, 1 mmol of PO_4 averages about 96.2 mg/dL.

E. Unit conversion for [Pi]: mg/dL × 0.3229 = mmol/L (SI unit, nearest 0.05 mmol/L)[11]

III. Hyperphosphatemia (Table 11.4)

A. Decreased urinary PO_4 excretion
1. Disorders that cause decreased GFR (see prerenal, renal, postrenal azotemia in Chap. 8): Hyperphosphatemia occurs because PO_4 is not filtered adequately from plasma; magnitude tends to parallel severity of azotemia in dogs, cats, and cattle, but it may not in horses.
2. Urinary bladder rupture or leakage of urine into tissues: Hyperphosphatemia results from decreased urinary excretion of PO_4 from the body.
3. Decreased [PTH] or activity (hypoparathyroidism or pseudohypoparathyroidism) (see Total Calcium Concentration, IV.B.1 above).
4. Acromegaly: GH increases tubular PO_4 resorption.[85]

B. Increased PO_4 absorption from intestine
1. Phosphate enema[87,88]

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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 (see Total Calcium Concentration, III.A.2).
3. Intestinal lesions requiring intestinal resection: Devitalized intestinal mucosa allows PO_4 to enter plasma (and peritoneal fluid); also shifting of PO_4 from ICF to ECF may be involved.[89]
4. Diet with low Ca^{2+}:PO_4 ratio

C. Shift of PO_4 from ICF to ECF
1. Myopathies (endurance rides in horses, exertional rhabdomyolysis, malignant hyperthermia): release of PO_4 from damaged muscle fibers[74-76,90]
2. Acute tumor lysis syndrome: release of PO_4 from necrotic neoplastic cells[77]

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, decreased [fCa^{2+}], and increased [PTH].[84]
2. Osteolytic bone lesions:[7] Some dogs with lymphoma and myeloma have localized bone resorption and corresponding hypercalcemia. Bone resorption may be due to cytokines or inflammatory mediators (e.g., IL-6, TGF, prostaglandins) produced by the cells, or to locally produced PTHrp (causing local resorption but not causing increased blood [PTHrp]). Other metastatic neoplasms are not recognized to cause hypercalcemia as commonly in domestic mammals as they do in people.

IV. Hypophosphatemia (Table 11.5)
A. Increased urinary PO_4 excretion

Table 11.5. Diseases and conditions that cause hypophosphatemia

Increased urinary PO_4 excretion
Prolonged diuresis
Increased [PTH] or [PTHrp] (see Table 11.2)
Fanconi syndrome (dogs)
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)
Glucose infusion
Respiratory alkalosis
Defective mobilization of PO_4 from bone
Postparturient paresis (milk fever) in cattle and eclampsia in bitches
Other or unknown mechanisms
*Equine renal disease (failure)
Halothane anesthesia in horses

Note: Conjugated bilirubin may interfere with some Pi assays to produce falsely increased or decreased results.

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B. Mg^{2+} is located in bones (about 60%), in soft tissues (about 38%), and in the extracellular fluid, including blood (1%-2%). Except in cattle, [tMg^{2+}] in erythrocytes is greater than in plasma (or serum).

C. Major factors that determine serum [tMg^{2+}]
   1. Hypoproteinemia causes a decrease in bound Mg^{2+} and thus may cause hypomagnesemia.
   2. Absorption of Mg^{2+} in gastrointestinal tract[7]
      a. In ruminants, Mg^{2+} is absorbed by the rumen (and maybe intestine) in a process linked to Na^+-K^+-ATPase.
      b. In monogastric animals, Mg^{2+} absorption occurs in the distal small intestine and colon and is enhanced by vitamin D and inhibited by high dietary Ca^{2+} or PO_4.
   3. Excretion
      a. Mg^{2+} in feces may represent unabsorbed dietary Mg^{2+} but also loss of endogenous Mg^{2+}.
      b. Kidneys
         (1) Mg^{2+} not bound to proteins freely passes through the glomerular filtration barrier; thus, decreased GFR can reduce renal excretion of Mg^{2+} and cause hypermagnesemia.
         (2) If the amount of Mg^{2+} entering the proximal tubules exceeds the tubular resorptive capacity, excess Mg^{2+} is excreted. PTH, osmotic diuresis, and loop diuretics increase renal excretion of Mg^{2+}.
         (3) If Mg^{2+} is present in bovine urine, the cow is not expected to be hypomagnesemic because the presence of Mg^{2+} typically indicates that transport maximum has been exceeded.
      c. Mammary gland during lactation: The ratio of milk to serum [tMg^{2+}] is about 5.

D. Hormonal regulation
   1. ADH, PTH, glucagon, calcitonin, and β-adrenergic agonists can stimulate Mg^{2+} absorption in the cortical thick ascending limb of the loop of Henle.[9]
   2. PTH can increase serum [tMg^{2+}] by increasing intestinal Mg^{2+} absorption and increasing Mg^{2+} resorption in renal tubules and bone.[97]
   3. Administration of 1,25-DHCC reduces plasma [tMg^{2+}], perhaps secondarily to decreased PTH activity.[97]
   4. Thyroxine tends to decrease plasma [tMg^{2+}] by increasing Mg^{2+} excretion in urine and feces.[98]
   5. Aldosterone promotes increased fecal and urinary Mg^{2+} excretion; aldosterone infusions may lead to decreased ruminal Mg^{2+} absorption.[99] Decreased aldosterone activity leads to increased serum [tMg^{2+}].

II. Analytical concepts of [tMg^{2+}] (serum or heparinized plasma)

A. Sample
   1. Serum is the preferred sample; heparinized plasma may be used in some assays.
   2. Blood anticoagulants that bind Mg^{2+} (e.g., EDTA, citrate, oxalate) should not be used in samples for [tMg^{2+}] assays.
   3. Except in cattle, *in vitro* hemolysis or delayed removal of serum from blood clot will cause increased serum [tMg^{2+}] because Mg^{2+} is liberated into serum from erythrocytes. In cattle, plasma and erythrocyte concentrations of Mg^{2+} are similar.

B. Clinical assays
   1. Common assays are photometric and measure [tMg^{2+}]. In these assays, metal-

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Table 11.6. Diseases or conditions that cause hypermagnesemia

Decreased urinary excretion
ªRenal failure and other causes of decreased GFR
Shift of fMg^{2+} from ICF to ECF
Active *in vivo* hemolysis
Increased intestinal absorption of Mg^{2+} without increased [PTH]
MgO, $Mg(OH)_2$, or similar antiacids/cathartics (cattle)
$MgSO_4$ (horses)
Other or unknown mechanisms
Milk fever
Excess intravenous infusion of Mg^{2+}

Note: Pseudohypermagnesemia may occur from delayed removal of serum from blood clot.

lochromic indicators or dyes change colors when they selectively bind Mg^{2+}. Atomic absorption spectrometry is the reference method but is not used in most clinical laboratories.

2. [fMg^{2+}] can be measured by ion-selective electrodes or it can be calculated from tMg^{2+} measurements before and after dialysis of serum to remove bound Mg^{2+}. However, there is currently very little clinical use of [fMg^{2+}] results.

C. Unit conversion: mg/dL × 0.4114 = mmol/L; mEq/L × 0.500 = mmol/L (SI unit, nearest 0.02 mmol/L)[11]

III. Hypermagnesemia (Table 11.6)
   A. Decreased urinary excretion: renal failure and other causes of decreased GFR
   B. Shift of Mg^{2+} from ICF to ECF: *In vitro* hemolysis, active *in vivo* hemolysis, or delayed removal of serum from blood clot will allow erythrocyte Mg^{2+} to be added to the serum concentration (except cattle).
   C. Increased intestinal absorption of Mg^{2+} without increased PTH
      1. Excess oral administration of Mg^{2+} in cattle (MgO, $Mg(OH)_2$)[101]
      2. Excess oral administration of Mg^{2+} in horses ($MgSO_4$)[102]
   D. Other or unknown mechanisms
      1. Milk fever: Increased PTH may induce increased renal resorption of Mg^{2+} leading to hypermagnesemia.[100]
      2. Excess intravenous infusion of Mg^{2+}
      3. In people, Addison's disease and hypothyroidism may cause hypermagnesemia. Similar findings have not been reported in domestic mammals.

IV. Hypomagnesemia (Table 11.7)
   A. Hypoproteinemia: decreased Mg^{2+} bound to proteins
   B. Inadequate ruminal or intestinal absorption of Mg^{2+}
      1. Prolonged anorexia or poor feed intake (especially in lactating cattle)
      2. Calves on whole milk diet[7]
      3. Grass tetany in cattle:[7,97]
         a. The hypomagnesemia is considered to result from decreased ruminal absorption of Mg^{2+}, but there are multiple theories for the absorption defect.
         b. Lush grass diet is high in PO_4 and K^+ content and low in Mg^{2+} and Na^+. In some

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B. PTHrp is produced by many cells in feti and adults, but plasma [PTHrp] is very low in healthy adults.

II. Analytical concepts
  A. Sample
    1. Because of rapid proteolysis of PTHrp, it is recommended that blood be collected into EDTA-tubes with an added protease inhibitor (aprotinin or leupeptin). Separated plasma is analyzed fresh or shipped frozen to reference laboratories.[108]
    2. Some laboratories, however, request serum samples be shipped on ice.
  B. Assays designed to measure human [PTHrp] (N-terminal) are used for measuring canine [PTHrp]. However, the same assay is not valid for equine [PTHrp].[17]

III. Increased [PTHrp] in serum (see Total Calcium Concentration, III.A.1)

VITAMIN D CONCENTRATION

I. Physiologic processes
  A. Formation of vitamin D
    1. Cholesterol is converted to 7-dehydrocholesterol, which is converted by ultraviolet light to cholecalciferol (vitamin D_3). Vitamin D_3 can also be of dietary origin. Vitamin D_2 (ergocalciferol) can be ingested in plants.
    2. 25-hydroxylase in hepatocytes catalyzes the formation of 25-HCC from cholecalciferol. 1α-hydroxylase in renal tubular cells catalyzes the formation of 1,25-DHCC from 25-HCC in most mammals. Studies have shown that horses lack renal 1α-hydroxylase and have low concentrations of 25-HCC; 24,25-DHCC; and 1,25-DHCC in plasma.[6]
    3. 1,25-DHCC (also called calcitriol) has 25 times the biologic activity of 25-HCC, which has 3–5 times the activity of cholecalciferol.
  B. Actions of vitamin D in dogs, cats, and cattle. (Roles of vitamin D in horses in regulation of [fCa^{2+}] appear to be minor.)[6]
    1. Vitamin D promotes intestinal uptake of Ca^{2+} by stimulating the formation of a Ca^{2+}-binding protein (calbindin) in the mucosal epithelial cells; it also stimulates absorption of PO_4.
    2. Vitamin D promotes Ca^{2+} and PO_4 liberation from bone by stimulating osteoclastic activity.
    3. Vitamin D promotes resorption of fCa^{2+} by proximal renal tubules by stimulating the formation of calbindin.
    4. Vitamin D inhibits PTH synthesis in parathyroid glands by inhibiting transcription of the PTH mRNA.
    5 Net effect of vitamin D: promotes hypercalcemia

II. Analytical concepts
  A. Sample
    1. Serum is the common sample but plasma (either heparin or EDTA) is acceptable; concentrations of 1,25-DHCC and 25-HCC are stable for 3 days at 24°C.[109] Samples should be frozen if analysis is delayed.
    2. Prior to most assays, the sample is deproteinized or extracted to free vitamin D metabolites from vitamin D-binding proteins (specific α-globulins and albumin). Nearly all vitamin D molecules are protein-bound in serum.

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II. Actions
  A. Calcitonin inhibits osteoclastic activity in bone.
  B. Calcitonin inhibits renal tubular resorption of Ca^{2+} and PO_4.
  C. Net effect of calcitonin activity: decreases serum [tCa^{2+}] and [Pi]

III. Analytical concepts
  A. Marked differences in amino acid sequences between species may limit the cross-species immunoreactivity in radioimmunoassays.
  B. Radioimmunoassays for canine calcitonin have been developed.[112,113]

IV. Increased calcitonin concentrations
  A. Medullary thyroid carcinoma[114]
  B. Nonthyroid cancer, especially from neural crest tissue

MAJOR PATTERNS FOR CALCIUM (Ca^{2+}) AND INORGANIC PHOSPHORUS (Pi) DISORDERS

Because of the physiologic relationships between Ca^{2+}, PO_4, PTH, PTHrp, and vitamin D, some diseases are expected to cause abnormal analyte concentrations and form patterns that suggest the presence of diseases. Patterns for major diseases or conditions are shown in Table 11.10. Because of compensation, some analyte concentrations may remain WRI but be inappropriately located near either the upper or lower limits of the reference interval.

Table 11.10. Expected hormone and mineral patterns for major diseases or conditions (without complications)

	[tCa^{2+}]	[Pi]	[PTH]	[PTHrp]	[1,25-DHCC]
Primary hyperparathyroidism	↑[a]	↓[b]	WRI-↑[c]	WRI	WRI
Humoral hypercalcemia of malignancy	↑	↓	↓-WRI	↑	WRI
Excess vitamin D	↑	WRI-↑	↓-WRI	WRI	↑
Canine hypoadrenocorticism	WRI-↑	WRI-↑	↓-WRI	WRI	WRI
Renal failure, hypercalcemic[d]	↑	WRI-↓	↓[e]	WRI	?[f]
Renal failure, chronic[g]	WRI-↓	↑	↑	WRI	↓-WRI
Hypoalbuminemia	↓	WRI	WRI	WRI	WRI
Primary hypoparathyroidism	↓	↑	↓	WRI	WRI
Milk fever	↓	↓	WRI-↑	WRI	WRI-↑
Prolonged anorexia	WRI	↓	WRI	WRI	WRI

Note: Secondary pathologic states may result in altered patterns. For example, prolonged or severe hypercalcemia may cause renal failure that might change serum [Pi] from hypophosphatemia to hyperphosphatemia.

[a] ↑ = above reference interval.

[b] ↓ = below reference interval.

[c] May not be above reference interval but is inappropriately high for the hypercalcemic status.

[d] More common in horses than other species, can be either acute or chronic renal failure.

[e] [PTH] is decreased in hypercalcemic renal failure in horses; similar data not found for other animals.

[f] Not known in most mammals; healthy horses have very little 1,25-DHCC and would expect less in the presence of hypercalcemia.

[g] More common form of renal failure in most domestic species

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Chapter 12

ENZYMES

Basic Principles in Clinical Enzymology 435
Alanine Transaminase (ALT) (Synonym Abbreviation: GPT) 443
Aspartate Transaminase (AST) (Synonym Abbreviation: GOT) 444
Lactate Dehydrogenase (LD) 445
Iditol Dehydrogenase (ID) (Synonym Abbreviation: SDH) 446
Alkaline Phosphatase (ALP) 446
γ-glutamyltransferase (GGT) (Synonym Abbreviation: GGTP) 450
Creatine Kinase (CK) 452
Amylase (AMS) 453
Lipase (LPS) 455
Other Serum Enzymes 456

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Table 12.1. Abbreviations and symbols in Chapter 12

»	symbol in tables to indicate relatively common disease or condition
ADP	adenosine diphosphate
ALP	alkaline phosphatase
ALT	alanine transaminase[a]
AMS	α-amylase
AST	aspartate transaminase[a]
ATP	adenosine triphosphate
B-ALP	bone alkaline phosphatase
C-ALP	corticosteroid-induced alkaline phosphatase
CK	creatine kinase
ECF	extracellular fluid
fCa^{2+}	free ionized calcium
fMg^{2+}	free ionized magnesium
GFR	glomerular filtration rate
GGT	γ-glutamyltransferase[b]
$(GGT:Crt)_u$	urine γ-glutamyltransferase activity to creatinine concentration
GOT	glutamate oxaloacetate transaminase[a]
GPT	glutamate pyruvate transaminase[a]
I-ALP	intestinal alkaline phosphatase
ICF	intracellular fluid
ID	iditol dehydrogenase
L-ALP	liver alkaline phosphatase
LD	lactate dehydrogenase
LPS	lipase
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
PO_4	inorganic phosphate (all forms)
PTH	parathyroid hormone
SDH	sorbitol dehydrogenase
SI unit	Système International d'Unités
tCa^{2+}	total calcium
TLI	trypsin-like immunoreactivity
U	international unit
URL	upper reference limit
USG_{ref}	urine specific gravity by refractometry
WRI	within reference interval

[a] NC-IUBMB recommended transaminase but considered aminotransferase acceptable.

[b] NC-IUBMB recommended γ-glutamyltransferase but considered γ-glutamyltranspeptidase (GGTP) acceptable.

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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* (or isozymes). If the different enzyme structure results from a posttranslational modification of an original gene product, then the proteins are *isoforms*.

I. Sources of serum enzymes (Table 12.2 and Fig. 12.1)
   A. Serum enzymes described in this chapter originate from cells (exogenous to serum) and do not have recognized functions in blood. Before release from cells, the serum enzymes may be in a cell's cytoplasm, mitochondria, or membrane. Serum enzyme activity in healthy animals is typically assumed to result from physiologic processes.

Table 12.2. Cellular or tissue sources and half-lives of common serum enzymes

Enzyme	Major mechanisms that lead to increased serum activity	Major cellular sources of increased serum enzyme activity	Half-lives[a]
ALP	Induction	»Hepatocytes (L-ALP) (C-ALP in dogs) »Biliary epithelium (L-ALP) Osteoblasts (B-ALP)	≈ 3 days (canine L-ALP)[71] < 8 hr (feline L-ALP)[72]
ALT	Cell damage	»Hepatocytes Skeletal myocytes	2–3 days (canine)[73]
AMS	Cell damage Decreased renal clearance	»Pancreatic acinar cells	≈ 5 hr (canine)[74]
AST	Cell damage	»Hepatocytes »Skeletal myocytes Cardiac myocytes Erythrocytes	7–8 days (equine)[75,76] < 1 day (canine)[77,78]
CK	Cell damage	»Skeletal myocytes Cardiac myocytes Smooth muscle myocytes (minor)	≈ 2 hr (equine)[75,76] < 2 hr (canine)[78]
GGT	Increased production	»Biliary epithelial cells »Hepatocytes	≈ 3 days (equine)[80]
ID	Cell damage	»Hepatocytes	≈ 4 hr (canine)[81]
LD	Cell damage	»Hepatocytes »Skeletal myocytes Cardiac myocytes Erythrocytes	< 6 hr (canine)[82]
LPS	Cell damage Decreased renal clearance	»Pancreatic acinar cells Liver neoplasms	≈ 2 hr (canine)[74]

[a] Data from available but limited sources.
([71-82] = numbers of sources in reference list.)

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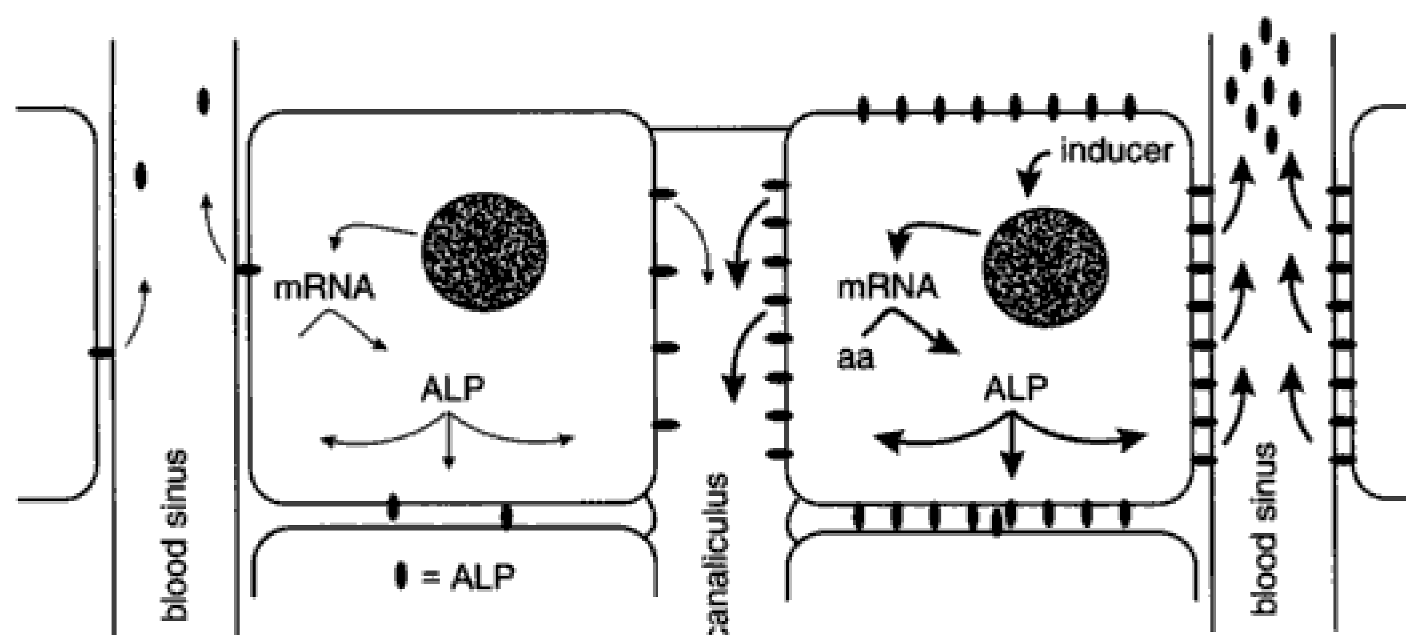

Fig. 12.3. Increased production of ALP by induction.
In healthy animals (*left half of drawing*), ALP is attached to hepatocyte membranes; more is located on the canalicular than the sinusoidal membrane. In sick animals (*right half of drawing*), drugs or metabolites (e.g., bile acid) induce the synthesis of more ALP that accumulates both on the canalicular and sinusoidal hepatocyte membranes. When more ALP is released from the sinusoidal membrane, serum ALP activity increases.

B. More enzymes produced by cells (primary mechanism for membrane enzymes, may be a mechanism for mitochondrial and cytoplasmic enzymes)
   1. Increased synthesis (production) of an enzyme usually is due to induction; that is, modifying transcription, translation, or other processes involving protein synthesis causes increased synthesis of the enzyme. Endogenous substances (e.g., bile acids) or drugs such as phenobarbital, prednisolone, or prednisone may trigger induction. Increases in serum ALP activity result from induction (Fig. 12.3).
   2. Increased synthesis may be due to hyperplasia or neoplasia of the enzyme's cell of origin.

C. Enzyme removal from plasma is decreased (enzyme has an increased half-life).
   1. Some enzymes (e.g., AMS and LPS) are inactivated or excreted by kidneys. Decreased renal blood flow leads to decreased inactivation of AMS and LPS.
   2. 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, there would be decreased removal of enzymes from plasma.

III. Decreased activity of most "serum enzymes" does not have diagnostic importance. Measured enzyme activity may be reduced because of poor sample handling (enzyme degraded), presence of an inhibitor (e.g., anticoagulants), reference interval that is not appropriate for the patient, or because of decreased mass of origin tissue.

IV. Measuring serum enzyme activity
   A. Enzymes are proteins that catalyze chemical reactions. Routine assays measure enzyme activity by detecting how fast a substrate is consumed or how fast a product forms.
      1. Enzyme assay theory: In the presence of excess substrate (S), the reaction rate is dependent on the quantity of enzyme (E); more specifically, the reaction rate is dependent on the rate of reaction from the enzyme-substrate complex to the product (P) + enzyme: $E + S \rightarrow E\text{-}S \rightarrow P + E$.

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2. Nearly all serum enzyme assays are spectrophotometric assays, either end-point or kinetic.
   a. End-point 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.
   b. 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).

B. Enzyme reactions for common clinical serum enzyme assays
   1. Fig. 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.
   2. Knowledge of the enzyme reactions promotes an understanding of enzyme nomenclature and physiologic roles of enzymes.

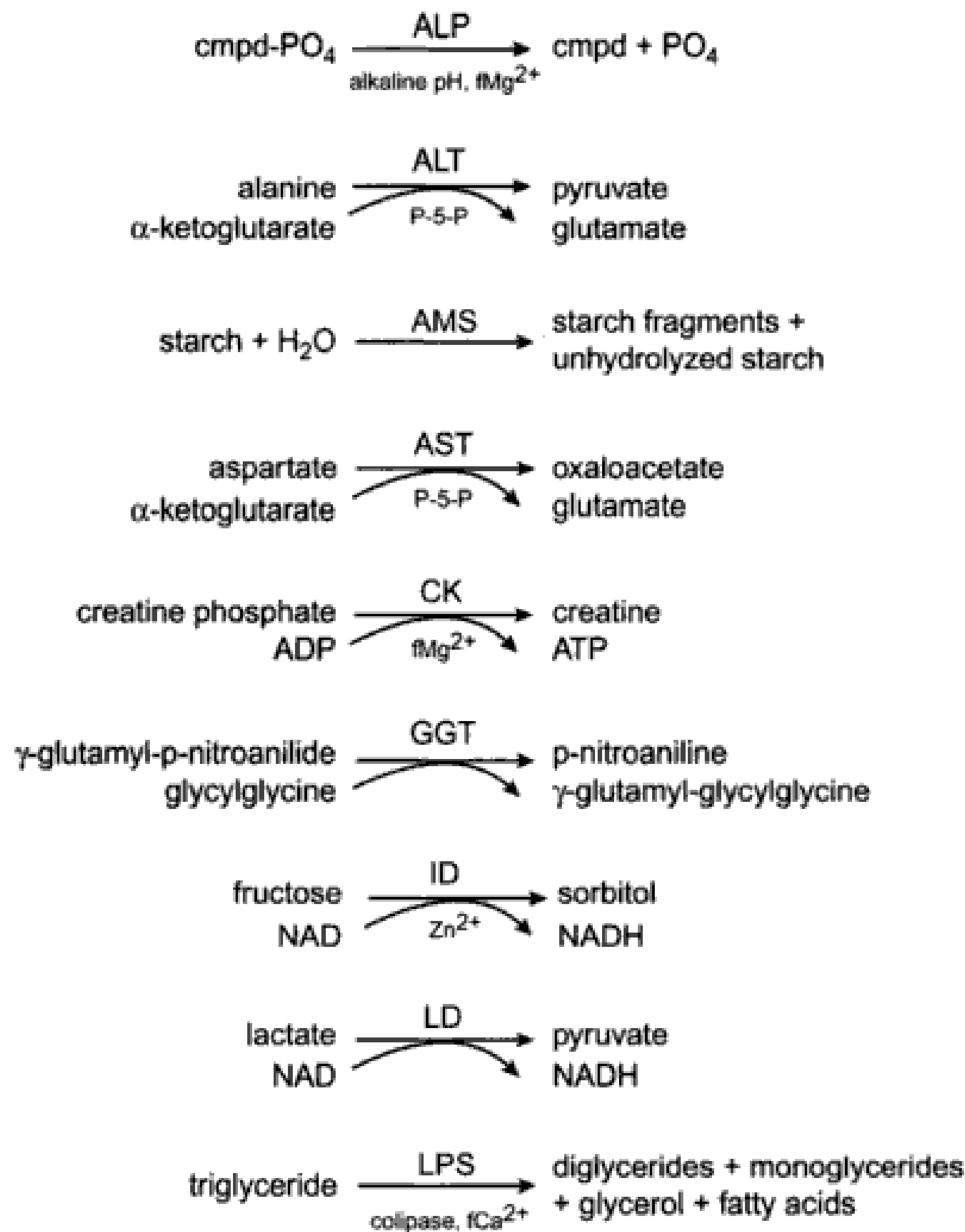

Fig. 12.4. Initial reactions in assays for the common clinical serum enzymes. (cmpd = compound) (P-5-P = pyridoxal-5′-phosphate)

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. LD activity can be assessed in reactions that are driven from lactate to pyruvate, or pyruvate to lactate.

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c. Hemoglobin may interfere with light transmission in spectrophotometric assays and give false increases or decreases depending on assay design.
2. Sera with elevated bilirubin (icteric sera): Bilirubin may interfere with light transmission.
3. Sera with increased visible lipoproteins (lipemic sera)
   a. Lipid molecules may interfere with light transmission.
   b. Lipid molecules cause unpredictable results because light transmission is hindered not only by number but also by size of molecules.

VII. Interpretation of increased serum enzyme activity
   A. Because of the variations in enzyme activity measured by different assays, patients' enzyme activities need to be compared to appropriate reference intervals. If increased, the degree of increase is usually determined by dividing the patient's enzyme value by the URL (the higher value of the reference interval) Example: patient's ALT = 500 U/L with a reference interval of 10–50 U/L; patient's ALT is 10 times the URL (10× URL). The absolute values for enzyme activities may vary considerably between assay methods, but the degree of increase above the URL should be nearly the same.
   B. The magnitude of increased enzyme activity may limit the possible explanations. For example, an ALT activity of 15× URL is probably due to 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 it 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 may return to reference interval sooner than AST activity.
   D. Integrate potential pathologic processes with other patient information to form ideas or explanations for the animal's illness.

VIII. Significance of increased serum enzyme activities
   A. Serum enzyme activities are markers or indicators of pathologic processes (e.g., hepatocyte injury, cholestasis) and not specific diseases. Many types of diseases may cause common pathologic processes.
   B. For the cytoplasmic enzymes (ALT, AMS, AST, CK, ID, LD, LPS), the magnitude of increase may relate to the severity of damage; i.e., slight damage may cause values < 2× URL, and severe damage might cause values > 50× URL. However, magnitude of increase does not differentiate reversible damage from irreversible damage, or local damage from diffuse damage.
   C. A mild increase (e.g., 2× URL) might not be very important in one animal because other findings clearly indicate a definite diagnosis. However, in some cases, the same serum enzyme activity might provide the only clue of active disease.
   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, there was a 9 times increase (10 times baseline value) associated with the disease. However, if the ALT reference interval was 20–70 U/L, the patient's 200 U/L value would represent only about 3x URL and might be considered a mild increase. Because pre-disease values are usually not available for individual animals, we must consider variations between animals when interpreting serum enzyme activity.
   E. Enzyme activity from a single sampling may not reflect the dynamic changes that may be

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Table 12.4. Disorders or conditions that cause increased serum ALT activity

- Hepatocyte damage (dogs and cats)
  - »Degenerative: hypoxia due to anemia or congestion
  - Anomalous: portosystemic shunt
  - »Metabolic: lipidosis, diabetes mellitus, feline hyperthyroidism
  - »Neoplastic: lymphoma and metastatic neoplasia, hepatocellular carcinoma
  - Nutritional: copper toxicosis, hemochromatosis
  - Inflammatory
    - »Infectious: leptospirosis, histoplasmosis, feline infectious peritonitis, bacterial cholangiohepatitis
    - »Noninfectious: chronic hepatitis, cirrhosis
  - Inherited: copper storage disease, lysosomal storage diseases
  - »Toxic: steroid hepatopathy, anesthetic agents, tetracycline, carprofen
  - »Traumatic: hit-by-car
- Induction[a]
  - »Phenobarbital
  - »Glucocorticoids: prednisone, prednisolone
- Skeletal muscle damage
  - Inherited: canine musculodystrophy
  - Traumatic: hit-by-car

Note: Lists of specific disorders or conditions are not complete but are provided to give examples.

[a] Increased serum activity is generally assumed to be due to induction in hepatocytes but such a cellular process is not proven for ALT activity. Increased activity could be due to a sublethal damage to hepatocytes caused by the drug or its metabolites.

URL).[12,13] Some dystrophic dogs occasionally have extreme ALT increases (20 times greater than 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).[14]

V. Species differences
   A. In dogs and cats, ALT is a major marker of hepatocyte damage but its serum activity is also increased by severe muscle disease.
   B. Hepatocytes of horses and cattle have so little ALT that ALT is not a useful marker of hepatocyte damage in these species.

ASPARTATE TRANSAMINASE (AST) (SYNONYM ABBREVIATION: GOT)

I. Physiologic processes, concepts, and facts: AST is a cytoplasmic and mitochondrial enzyme that catalyzes a reversible reaction involved in the deamination of aspartate to form oxaloacetate, which can enter the Kreb's cycle.

II. Tissue sources of increased serum AST activity and AST half-life (Table 12.2)

III. Analytical concepts: Other than variations in assay temperatures, there are minimal variations in serum AST activity between assay systems if results are reported in U/L.

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III. Analytical concepts
   A. Variations in assay temperatures can alter LD activity.
   B. Some assays measure LD activity in a pyruvate to lactate reaction; others measure LD activity in a lactate to pyruvate reaction. The LD activity in the two types of reactions can vary considerably,[17] perhaps because LD-1 is inhibited by high pyruvate concentrations and LD-5 maintains activity at high pyruvate concentrations.[18]

IV. Increased serum LD activity (Table 12.5)
   A. In all species, LD is a marker of hepatocyte damage but serum LD activity is also increased by muscle damage and hemolysis.
   B. Increased LD activity may be due to reversible or irreversible, focal or diffuse cell damage.
   C. By itself, serum LD activity is a screening test for hepatocyte or muscle damage. In a group of tests, LD activity provides additional information that may help explain activities of more tissue-specific enzymes (i.e., ALT, ID, CK).

IDITOL DEHYDROGENASE (ID) (SYNONYM ABBREVIATION: SDH)

I. Physiologic processes, concepts, and facts
   A. ID is a cytoplasmic enzyme that catalyzes a reversible reaction involving conversion of fructose to sorbitol (or glucitol).
   B. The established name for the enzyme is iditol dehydrogenase, but iditol is not a substrate or product in clinical assays. In the clinical assay, fructose is the substrate.

II. Tissue sources of increased serum ID activity and ID half-life (Table 12.2)

III. Analytical concepts
   A. Other than variations in assay temperatures, there are minimal variations in serum ID activity between assay systems if results are reported in U/L.
   B. ID assays are not common components of serum chemical profiles, probably because the enzyme's stability is not sufficient for routine sample handling.

IV. Increased serum ID activity
   A. Indicates hepatocyte damage; may be reversible or irreversible, focal or diffuse; unusual to find ID activity > 10× URL
   B. ID activity is used primarily in horses and cattle because other common hepatic cytosolic enzymes (AST, LD) are not liver-specific and ALT is not a useful marker of hepatocyte damage in horses and cattle.
   C. ID activity could be used as a marker of hepatocyte damage in dogs and cats, but ALT assays are more commonly available and the ALT is not as labile.

ALKALINE PHOSPHATASE (ALP)

I. Physiologic processes, concepts, and facts
   A. ALP includes a family of phosphatases that have phosphatase activity in an alkaline environment. Another family has phosphatase activity in an acid environment (the acid phosphatases).
   B. Physiologic roles of ALP are not known but the enzymes are associated or attached to

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reasons for increased glucocorticoid hormones and thus increased C-ALP. Also, dogs with excess glucocorticoid hormones may have increased activity of both L-ALP and C-ALP.

D. 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 sera of two healthy dogs.[30]
   2. Increased total ALP and B-ALP activities in frozen sera from dogs with osteosarcoma have been associated with shorter patient survival. The degrees of increases in ALP and B-ALP were not reported.[33]
   3. Cats with hyperthyroidism have increased B-ALP activity that causes increased total ALP activity.[34]

E. Benign familial hyperphosphatasemia in Siberian huskies[35]
   1. Of 42 pups in eight related litters of Siberian huskies, 17 pups had serum ALP activities about 6 times the activities found in other age-matched Siberian huskies. B-ALP was the isoform causing increased total ALP activity in all 5 of the puppies for which isoforms were assessed.
   2. The cause of the increased ALP was not determined. Serum concentrations of tCa^{2+}, PO_4, and PTH were not different from matched pups.

F. Pregnant women may have an increased serum ALP activity due to increased placental ALP. ALP activity did not increase in pregnant mares.[36] The increase in serum ALP during pregnancy in bitches is too small to affect ALP interpretation.[37] Placental ALP may contribute to serum ALP activity in late-term pregnancy of cats.[38]

V. Species differences

A. Dogs
   1. ALP has high diagnostic sensitivity for detecting cholestasis. ALP activity may be increased before icterus appears. ALP values may range from < 2× URL to > 20× URL.
   2. Increased ALP activity induced by corticosteroids may result from induced synthesis of L-ALP and C-ALP; ALP values may range from < 2× URL to > 20× URL.[28]
   3. Phenobarbital, primidone, and phenytoin are described in clinical studies as either inducing the synthesis of L-ALP or causing hepatocellular damage in dogs.[39]
   4. Increased ALP activity due to increased osteoblastic activity in growing dogs (production of B-ALP) is typically mild (< 4× URL).

B. Cats
   1. ALP has poor diagnostic sensitivity for detecting cholestasis. Cats typically are icteric before ALP activity increases. ALP values may range from < 2× URL to > 10× URL.
   2. It has been reported that from 43%–75% of hyperthyroid cats have increased serum ALP activities. ALP activities typically are < 4× URL. In such cats, the ALP activity is due to L-ALP and B-ALP, whereas ALP activity in healthy mature cats is due to L-ALP. Some hyperthyroid cats have increased B-ALP activity in serum without a concurrent increase in total ALP activity.[40]

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.

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2. In horses with colic or intestinal lesions, ALP activity may increase in the peritoneal fluid due to increased granulocytic or intestinal ALP activity. However, granulocytic or intestinal ALP did not increase total ALP activity in serum.[41,42]

D. Cattle: ALP has moderate diagnostic sensitivity for detecting cholestasis but cholestasis disorders are uncommon in cattle.

γ-GLUTAMYLTRANSFERASE (GGT) (SYNONYM ABBREVIATION: GGTP)

I. Physiologic processes, concepts, and facts
   A. GGT is associated with cell membranes. It catalyzes the transfer of glutamyl groups between peptides, and it is involved in glutathione reactions. Many cells have GGT activity, but biliary epithelial cells, pancreas, 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.[43,44]
   B. Colostrum of cows has high GGT activity and the GGT molecules may be absorbed from the calf intestine after colostral intake. Postsuckling calves may have GGT activities as high as 20× URL (using adult reference intervals) and up to 16 times presuckling values.[45-47] This physiologic change can be used as evidence of suckling and thus as an indication of successful passive transfer.
   C. Mare colostrum contains relatively little GGT activity and GGT activity in neonatal foals does not increase after suckling. However, serum GGT activity in foals less than 1 month old is about 1.5–3 times as great as the GGT activity in adult horses.[48]
   D. Because of high colostral GGT, serum GGT activity in 1- to 3-day-old pups is up to 100× URL (using adult reference intervals) but returns to presuckle values within 10 days after suckling.[19]

II. Tissue sources of increased serum GGT activity and GGT half-life (Table 12.2)

III. Analytical concepts
   A. Other than variations of assay temperatures, there are minimal variations in serum GGT activity between assay systems if results are reported in U/L.
   B. In a study involving rat samples, heparin was shown to nearly double the GGT activity in an assay that used γ-glutamyl-p-nitroanilide as a substrate.[6,49] Heparin also can cause turbidity in the reaction fluid, which would interfere with transmission photometry.[17] There were no differences in GGT activities between canine serum and heparinized plasma samples using the dry-slide method (Vitros®) that uses the same substrate (unpublished data).

IV. Increased serum GGT activity (Table 12.7)
   A. Cholestasis or biliary hyperplasia
      1. Cholestasis is expected to cause increased hepatic and plasma bile acid concentrations. Increased bile acids or other constituents of bile may stimulate the synthesis and release of GGT, but mechanisms of increase are not established.[23] Disorders that result in cholestasis may also induce biliary hyperplasia and a resultant increase in GGT activity.
      2. Experimental data indicate that increased serum GGT activity is primarily dependent on the degree of hyperplasia of biliary epithelial cells and not on induction of GGT synthesis, hepatocyte damage, or cholestasis.[50]

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Table 12.7 Disorders or conditions that cause increased serum GGT activity

»Cholestasis (see cholestasis conditions listed for increased ALP in Table 12.6)
Biliary hyperplasia (pyrrolizidine alkaloid-containing plants)
Induction by drugs or hormones
Phenobarbital, dilantin, primidone
Corticosteroids: endogenous or exogenous[a]

Note: Ingestion and absorption of colostrum by neonatal calves and pups may increase serum GGT activity.

[a]Increased serum GGT activity is generally assumed but has not been proven to be due to induction.

B. Associated with drugs or hormones
   1. Serum GGT activities increased (2–3 times prevalues) transiently (from 13 to 17 weeks) in dogs given phenobarbital for 27 weeks, but mean activity did not exceed reference intervals.[51]
   2. Increased serum GGT activity in dogs being treated with glucocorticoid hormones may not be due to induction, since glucocorticoids did not induce GGT synthesis in cultured hepatocytes.[11] However, in prednisone-treated dogs, there were increases in both hepatic and serum GGT activities which suggested induction or effects of steroid hepatopathy.[28]

V. Species differences
   A. Horses
      1. Increased GGT activity has better diagnostic sensitivity than ALP for detecting cholestasis or other biliary disorders in horses.
      2. Neonatal foals have greater serum GGT activity than do their mares.[48]
   B. In dogs, increased GGT activity tends to parallel increases in ALP activity due to cholestatic disorders, but ALP probably has more diagnostic sensitivity; GGT values can be increased in dogs with steroid hepatopathy.
   C. In 54 cats with liver diseases, disorders associated with increased GGT activity included bile duct obstruction, cholangitis, cholangiohepatitis, lipidosis, neoplasia, hepatic necrosis, and cirrhosis; GGT activity in 9 (17%) of the cats was WRI.[52] In 12 of 15 (80%) of the cats with lipidosis, the ALP:GGT ratio was increased (i.e., ALP activity increased more than GGT activity). Only 4 of 39 (10%) of the cats with liver diseases other than lipidosis had increased ALP:GGT ratios (magnitudes of change not reported).

VI. GGT activity in urine
   A. Damage to renal epithelial cells causes an increased urinary excretion of renal GGT without increased serum GGT activity.
   B. Because the urinary GGT activity is dependent 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 Chap. 8).
      1. An increased $(GGT:Crt)_u$ ratio should indicate that the rate of GGT excretion is increased relative to the rate of creatinine excretion. Such an increased $(GGT:Crt)_u$ ratio has occurred with renal diseases in dogs and ponies.[53]
      2. Because creatinine clearance is almost entirely dependent on GFR but GGT excre-

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tion is not, it is possible that an increased $(GGT:Crt)_u$ ratio could be due to decreased GFR and not increased release of GGT by tubules.

3. The units for reported $(GGT:Crt)_u$ ratios have been mixed volume units; i.e., calculated from GGT in U/L and Crt in mg/dL.

CREATINE KINASE (CK)

I. Physiologic processes, concepts, and facts
   A. CK is a cytoplasmic enzyme that catalyzes a reversible reaction involved in the transfer of PO_4 from creatine-PO_4 to ADP to form ATP. Creatine phosphokinase (CPK) is not an acceptable name for the enzyme.
   B. CK is a dimer. There are four isoenzymes that have variable cell distributions: CK-1 dominates in brain, CK-2 and CK-3 in cardiac and skeletal muscle, and CK-Mt in mitochondria of many tissues.

II. Tissue sources of increased serum CK activity and CK half-life (Table 12.2)

III. Analytical concepts: Other than variations in assay temperatures, there are minimal variations in serum CK activity between assay systems if results are reported in U/L. The analytical ranges of some commercial assays are too narrow for some domestic mammals and thus sera frequently may need to be diluted to obtain numeric results.

IV. Increased serum CK activity (Table 12.8)
   A. A variety of insults (pathologic and iatrogenic) may damage muscle fibers and cause the release of CK from muscle fibers. CK may be released because of necrosis or reversible cell damage.
      1. The magnitude of increase can be mild to extreme (< 2× URL to > 50× URL) and is somewhat proportional to the degree of muscle damage.
      2. From a single insult (e.g., recumbency or other trauma), there can be very rapid increase (hours) and a rapid decline (hours to days) because of the short CK half-life.

Table 12.8. Disorders or conditions that cause increased serum CK activity

Muscle damage (mostly skeletal, occasionally cardiac, rarely smooth)
[a]Degenerative: hypoxia due to exertion or seizures, exertional rhabdomyolysis, saddle thrombus
Metabolic: feline hyperthyroidism
Neoplastic: metastatic neoplasia
Nutritional: vitamin E or Se deficiency
Inherited: musculodystrophy, hyperkalemic myopathy
Inflammatory: myositis due to *Neospora*, *Toxoplasma*, bacteria or other agents
Toxic: monensin, castor bean, gossypol
[a]Traumatic: intramuscular injections, hit-by-car, recumbency in horses and cattle, seizures, exertion

Note: Lists of specific diseases or conditions are not complete but are provided to give examples. *In vitro* hemolysis may cause a falsely increased CK activity in some CK assays.

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Table 12.9. Disorders or conditions that cause increased serum AMS and LPS activities

Pancreatic acinar cell damage: inflammation, neoplasia (AMS and LPS)
Decreased renal clearance
Prerenal disorders: dehydration, shock (AMS and LPS)
Renal disorders: acute or chronic renal diseases (AMS and LPS)
Postrenal disorders: urinary tract obstruction (AMS and LPS)
Macroamylasemia (AMS)
Other or unknown mechanism
Dexamethasone treatment (LPS)
Hepatic neoplasia (LPS)

not result in acinar cell necrosis. The AMS may gain access to blood via lymphatic vessels. In experimental canine pancreatitis, serum AMS activity peaks within 12–48 hr and persists for 8–14 days.[57]

2. Species variations
   a. Serum AMS activity in dogs with acute pancreatitis range from WRI to extremely increased (> 10× URL).
   b. In cats with spontaneous pancreatitis, serum AMS activity may be WRI or mildly increased (< 3× URL).[58,59] In experimental pancreatitis in six cats, serum AMS activity did not increase.[60]
   c. Measurement of serum AMS activity has not been useful for diagnosing pancreatitis or other diseases in horses or cattle.
3. Pancreatic neoplasia can also lead to increased serum AMS activity.

B. Decreased renal inactivation or excretion
   1. In dogs, clinical and experimental data indicate that there is an increased half-life of plasma AMS when there is decreased renal blood flow or decreased functional renal tissue.[61,62] Experimentally, the increased half-life may lead to hyperamylasemia (mean increase of 2.5–4.0 times baseline values). Clinically, the increased AMS activity is expected to be < 3× URL in conditions that cause decreased GFR without pancreatic acinar cell damage.
   2. Exactly how renal functions influence serum AMS activity has not been established.
      a. Very little to no AMS activity is found in the urine of healthy dogs.
      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 AMS that gets into the tubular lumen, or perhaps a large amount of luminal protein interferes with AMS inactivation by the tubular epithelial cells.[56,63,64]
      c. Experimental damage to proximal renal tubular cells causes increased urinary loss of AMS, but relatively not as much as urinary loss of LPS or lysozyme. Proximal tubular epithelial cells have some ability to inactivate or resorb AMS if AMS does get through the glomerular filtration barrier.[65]
      d. Formation of macroamylase molecules in blood also influences serum AMS activity, as they would not pass through a healthy glomerular filtration barrier and may have a longer half-life (macroamylase is a complex between AMS and an immunoglobulin or other protein). Macroamylasemia contributes to the hyperamylasemia in some dogs with renal failure. However, hyperamylasemia was still present in some sera after the macroamylase molecules were removed.[56]

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line values). Clinically, the increased LPS activity is expected to be < 4× URL in conditions that cause a decreased GFR without pancreatic acinar cell damage. However, greater LPS activity (6–8× URL) has been reported with some renal cases that did not have evidence of pancreatic disease[68] and 10× URL in a case of spontaneous primary renal failure.[62]

2. Hyperlipasemia associated with renal disease may be due to decreased renal inactivation of LPS.

C. Associated with pancreatic or hepatic neoplasia in dogs: In five of six cases, histochemical and immunohistochemical findings indicated that pancreatic and hepatic neoplasms were potential sources of mild to marked increased serum LPS activity.[69] The same dogs did not have significant increases in serum AMS activity.

D. Associated with dexamethasone treatments in dogs[70]

1. Hyperlipasemia (< 2× URL) was found in 24 healthy dogs by the 8th day 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 = 4.6× URL) occurred by the 10th day of treatment (initial dose at 2 mg/kg then reduced doses); the number of sampled dogs varied from two to eight on different days.
2. Microscopic evidence of pancreatic damage was not found and concurrent hyperamylasemia was not found.

OTHER SERUM ENZYMES

Many serum enzymes have been assessed in an attempt to find better indicators of pathologic states involving hepatocytes or other cells (Table 12.10). For a variety of reasons, most have failed to be as clinically valuable as those described earlier in this chapter.

Decreased TLI has shown to be valuable in diagnosing exocrine pancreatic insufficiency. Increased TLI has been found in active pancreatitis (see Chap. 15).

Table 12.10. Other serum enzymes

Serum enzyme	Disorder causes	Diagnostic value
5′-nucleotidase	↑	Hepatobiliary disease
Aldolase	↑	Muscle fiber damage
Arginase	↑	Hepatocyte damage
Cholinesterase[a]	↓	Organophosphate toxicosis, infections, chronic liver disease
Glutamate dehydrogenase	↑	Hepatocyte damage
Isocitrate dehydrogenase	↑	Hepatocyte damage
Leucyl aminopeptidase[b]	↑	Hepatobiliary disease
Maltase	↑	Intestinal mucosa damage
Muramidase (lysozyme)	↑	Neoplasia (histiocytic)
Malate dehydrogenase	↑	Hepatocyte damage
Ornithine carbamoyl-transferase	↑	Hepatocyte damage
Trypsin-like immunoreactivity	↑	Pancreatitis or decreased GFR
(TLI) (see Chap. 15)	↓	Exocrine pancreatic insufficiency in dogs and cats

[a] Plasma "pseudocholinesterase" or butyrylcholinesterase; not acetylcholinesterase of myoneural junction.
[b] Also called leucine aminopeptidase.

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Table 13.1. Abbreviations and symbols in Chapter 13

»	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
ADP	adenosine diphosphate
ALP	alkaline phosphatase
ALT	alanine transaminase
AMP	adenosine monophosphate
AST	aspartate transaminase
ATP	adenosine triphosphate
Bδ	delta bilirubin
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)
Epo	erythropoietin
FDP	fibrin or fibrinogen degradation product
GGT	γ-glutamyltransferase
Hct	hematocrit
Hgb	hemoglobin
ID	iditol dehydrogenase
IL-6	interleukin-6
LD	lactate dehydrogenase
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NH_3	ammonia
NH_4^+	ammonium
PT	prothrombin time
PTT	partial thromboplastin time
SI unit	Système International d'Unités
TNFα	tumor necrosis factor-α
TP	total protein
USG_{ref}	refractometric urine specific gravity
WRI	within reference interval

PHYSIOLOGIC FUNCTIONS OF THE LIVER

The liver has many vital physiologic functions involving synthesis, excretion, and storage. When a disease process damages cells within a liver, changes in hepatic function may result in abnormal composition of body fluids that can be detected by laboratory assays.

I. Functions involving body fuels
   A. Protein synthesis: Hepatocytes synthesize most plasma proteins (over 1000 proteins), including albumin and most globulins (except immunoglobulins). Most synthesis is *de novo* (from new), either from essential (dietary) amino acids or from nonessential amino acids made by hepatocytes.

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Table 13.2. CBC and chemistry test results (pathologic findings) that suggest or indicate hepatic disease or dysfunction

Pathologic findings	Hepatic lesion suggested	Pathogenesis of finding[a]
CBC results		
Acanthocytosis	Hemangiosarcoma	Possibly vascular trauma
	Lipid metabolism defect	Altered lipid composition of erythrocyte membrane
Anemia	Hepatitis	Anemia of inflammatory disease
	↓ functional mass[b]	Possibly ↓ Epo or abnormal protein or amino acid metabolism
Codocytosis	↓ functional mass	Altered lipid composition of erythrocyte membrane
Microcytosis	↓ functional mass	Possibly ↓ transferrin production and thus ↓ delivery of iron to erythrocyte precursors
	Portosystemic shunt	
Chemistry assay results		
↓ UN	↓ functional mass	↓ urea production
Hyperammonemia	↓ functional mass	Inadequate fixing of NH_4^+ into urea
	Portosystemic shunt	
Hyperbilirubinemia	Cholestasis	Inadequate biliary excretion of bilirubin
	↓ Bc transport	
Hypercholesterolemia	Cholestasis	↑ production of cholesterol and ↓ clearance of lipoproteins
Hyperglycemia	Cirrhosis	Hyperglucagonemia or increased gluconeogenesis of hepatocutaneous syndrome
	Hepatopathy	
Hyperuricemia	↓ functional mass	↓ conversion of uric acid to allantoin by hepatocytes
Hypoalbuminemia	↓ functional mass	↓ albumin production
Hypocholesterolemia	↓ functional mass	↓ cholesterol synthesis
Hypofibrinogenemia	↓ functional mass	↓ fibrinogen production
Hypoglycemia	↓ functional mass	↓ gluconeogenesis
Hypoproteinemia	↓ functional mass	↓ production of albumin and globulins other than γ-globulins
↑ ALT, AST, ID, LD activities	Damaged hepatocytes	Release of cytoplasmic enzymes due to blebbing or necrosis
↑ ALP activity	Cholestasis	↑ production of L-ALP
↑ GGT activity	Biliary hyperplasia	↑ production of GGT
Lipemia (gross)	↓ functional mass	↓ clearance of lipoproteins

[a] See more complete explanations in chapters for each particular analyte.

[b] Decreased functional mass results from diffuse hepatocyte damage, destruction, atrophy, or hypoplasia.

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Table 13.3. Urinalysis, coagulation, fecal, and peritoneal fluid test results (pathologic findings) that suggest or indicate hepatic disease or dysfunction

Pathologic findings	Hepatic lesion suggested	Pathogenesis of finding[a]
Urinalysis results		
Ammonium biurate crystalluria[b]	↓ functional mass	Inadequate fixing of NH_4^+ into urea and ↓ conversion of uric acid to allantoin
Bilirubinuria[b]	Cholestasis ↓ Bc transport	Inadequate biliary excretion of bilirubin
Hyposthenuria or isosthenuria[b]	↓ functional mass	↓ renal medullary tonicity due to decreased urea concentration; ↑ NH_4^+ excretion may inhibit concentrating mechanism
Urate crystalluria[b]	↓ functional mass	↓ conversion of uric acid to allantoin
Coagulation assay results		
Prolonged PTT or PT	Cholestasis	↓ vitamin K-dependent coagulation factors due to impaired intestinal absorption of vitamin K
	↓ functional mass	↓ clearance of inhibitors of coagulation factors such as FDP ↓ production of most coagulation factors
Fecal exam results		
Steatorrhea	Cholestasis	Defective lipid digestion because bile acids not delivered to intestine
Peritoneal fluid analysis		
Transudate	↓ functional mass Cirrhosis	↑ Na^+ and H_2O retention, ↓ plasma oncotic pressure, portal hypertension, ↓ lymph drainage

[a] See more complete explanations in chapters for each particular analyte.

[b] May occur in clinically healthy animals; e.g., ammonium biurate crystalluria in dalmations, urate crystalluria in English bulldogs, bilirubinuria in dogs, and hyposthenuria or isosthenuria as a physiologic response to excess water intake.

B. Biliary disease may also result from a variety of insults, but it is most often the result of inflammatory, neoplastic, or toxic disorders of the biliary system.

C. Hepatic insufficiency is a pathophysiologic state in which there is a marked reduction of functioning hepatocytes (hepatic insufficiency and hepatic failure are usually considered synonyms). Disorders that cause hepatic insufficiency fall into two groups:
   1. Disorders that destroy hepatocytes (hepatocellular disease) may progress slowly, recur episodically, or involve rapid, extensive necrosis.
   2. Portosystemic shunts (congenital or acquired) cause either hypoplasia or atrophy of liver because of decreased nutrients reaching hepatocytes from portal blood.

D. All animals with hepatic insufficiency have too few functioning hepatocytes, but many animals with hepatocellular disease do not have hepatic insufficiency. Most animals with primary biliary disease will develop secondary hepatocellular disease. Many animals with primary hepatocellular disease develop secondary biliary disorders. Either hepatocellular or biliary diseases may result in hepatic insufficiency.

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E. Laboratory test results that indicate hepatic insufficiency do not tell us which hepatocellular or hepatobiliary disease the animal has, or if the disease process is reversible or irreversible.

III. In clinical jargon, some people refer to hepatic enzymes (ALT, AST, ALP, GGT, ID) as liver function tests. However, increased activities of the enzymes do not directly indicate loss of any liver function, and liver function can be greatly reduced without increased serum enzyme activities. To emphasize the latter concept, would the serum activity of a hepatic enzyme increase if you removed an animal's liver?

IV. Decreased functional hepatic mass
  A. Hepatic insufficiency occurs when hepatic functional mass has decreased enough to cause a pathophysiologic state. The decreased functional mass typically results from too few hepatocytes (thus a small liver). However, it also may result from dysfunction of existing hepatocytes and occur with hepatomegaly or a normal-sized liver.
  B. There are many diseases and conditions that may decrease an animal's functional hepatic mass. These include the following:
    1. Degenerative: hypoxia due to anemia or congestion
    2. Anomalous: portosystemic shunt
    3. Metabolic: lipidosis, diabetes mellitus, hyperadrenocorticism
    4. Neoplastic: lymphoma, metastatic neoplasia
    5. Inflammatory
      a. Infectious: leptospirosis, histoplasmosis, feline infectious peritonitis, bacterial cholangiohepatitis, Tyzzer's disease
      b. Noninfectious: chronic active hepatitis
    6. Inherited: copper or lysosomal storage diseases
    7. Toxic: steroid hepatopathy, some anesthetic agents, tetracycline, carprofen, copper toxicosis, hemochromatosis, pyrrolizidine alkaloid-containing plants
    8. Traumatic: hit-by-car

V. Cholestasis
  A. Cholestasis is defined in different ways. In a dictionary definition, cholestasis is the "stoppage or suppression of bile flow."[2]
    1. The "stoppage" component of the definition is consistent with the observations of distended bile ducts (grossly) and bile plugs, lakes, or pigments (microscopic) that result from extrahepatic or intrahepatic lesions that obstruct bile flow and thus reduce the volume of bile leaving the biliary system.
    2. The "suppression" component of the definition is consistent with pathophysiologic states involving impaired BA excretion into bile. As the osmotic effects of secreted BA largely control bile volume, decreased BA secretion may reduce the volume of bile produced by the biliary system and thus reduce bile flow (less bile volume/day).
    3. *Cholestasis* is commonly used in the context of the icterus that occurs because of obstructive biliary lesions. However, Bc is a very small component of bile and contributes little to formation of bile or bile volume. When there is obstructive biliary disease, there probably is decreased bile flow, decreased Bc excretion, and decreased BA excretion.
    4. From one perspective, understanding "obstructive cholestasis" is necessary to explain structural consequences of biliary disease. However, from another perspective, under-

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standing "functional cholestasis" is necessary to explain the pathophysiologic consequences of defective bile acid excretion.

B. The different uses of the term *cholestasis* are mentioned to help clarify statements that may appear to be conflicting. For example, a pathophysiologic state associated with endotoxemia is called either "functional cholestasis" or "sepsis-associated cholestasis." In this context, there is defective BA excretion from hepatocytes to bile but there is not an obstructive lesion that impairs bile flow.

C. Bile acid and bilirubin pathways have similarities but also marked differences.[3] They will be covered in more detail in separate sections.
   1. Bu is a product of heme degradation; BA is a product of cholesterol degradation. Heme and cholesterol do not share physiologic pathways.
   2. Bilirubin (Bc and Bu) and BA enter hepatocytes through sinusoidal membranes, but by different membrane transport systems.
   3. Bc and BA are excreted from hepatocytes through canalicular membranes, but by different membrane transport systems.
   4. Pathologic states that damage hepatocyte membranes can result in both impaired bilirubin and BA excretion. These defects may be further linked because accumulation of BA in hepatocytes may cause hepatocyte damage that interferes with bilirubin metabolism. However, pathologic states that selectively interfere with transport systems may result in impaired excretion of one analyte but not the other.

BILIRUBIN CONCENTRATION

I. Physiologic processes (Fig. 13.1)

A. In plasma, there are three fractions of total bilirubin (Bu/Alb, Bc, Bδ-Alb); Bu/Alb is dominant in the absence of disease. Bu is constantly produced from the turnover of heme from senescent erythrocytes and heme-containing proteins. In health, Bu and Bc are rapidly removed from plasma by either liver (Bu, Bc) or kidneys (Bc). The spontaneous and covalent binding of bilirubin glucuronide isomers (Bc) to albumin forms Bδ; once formed, it has the circulating half-life of albumin (≈ 10–14 days).[4]

B. Small amounts of bilirubin are commonly found in urine of healthy dogs. It has not been established if the bilirubin is Bc or Bu/Alb. It is frequently assumed to be Bc because Bc is H_2O soluble and passes freely through the glomerular filtration barrier. However, small amounts of albumin are also commonly present in urine of healthy dogs, so some bilirubin may be in the form of Bu/Alb.

C. Healthy horses have higher serum [Bt] than other domestic animals, about 0.7–2.0 mg/dL in horses versus < 0.5 mg/dL in other animals.

II. Analytical concepts

A. Unit conversion: mg/dL × 17.10 = μmol/L (SI unit, nearest 2 μmol/L)[5]

B. Conventional spectrophotometric methods (wet chemical assays)
   1. There are several bilirubin assays that attempt to measure all bilirubin fractions (total bilirubin, Bt) or only the non-Bu fractions (direct bilirubin, Bd). The classic bilirubin assay was the van den Bergh method, but it has been replaced by other assays (e.g., Malloy-Evelyn and Jendrassik-Gróf methods) for several decades.
      a. Bu reacts slower than Bc in most bilirubin reactions, so substances are added to accelerate its participation in [Bt] assays. When the accelerant factors are not present, then the non-Bu fractions are measured (called direct bilirubin reaction).

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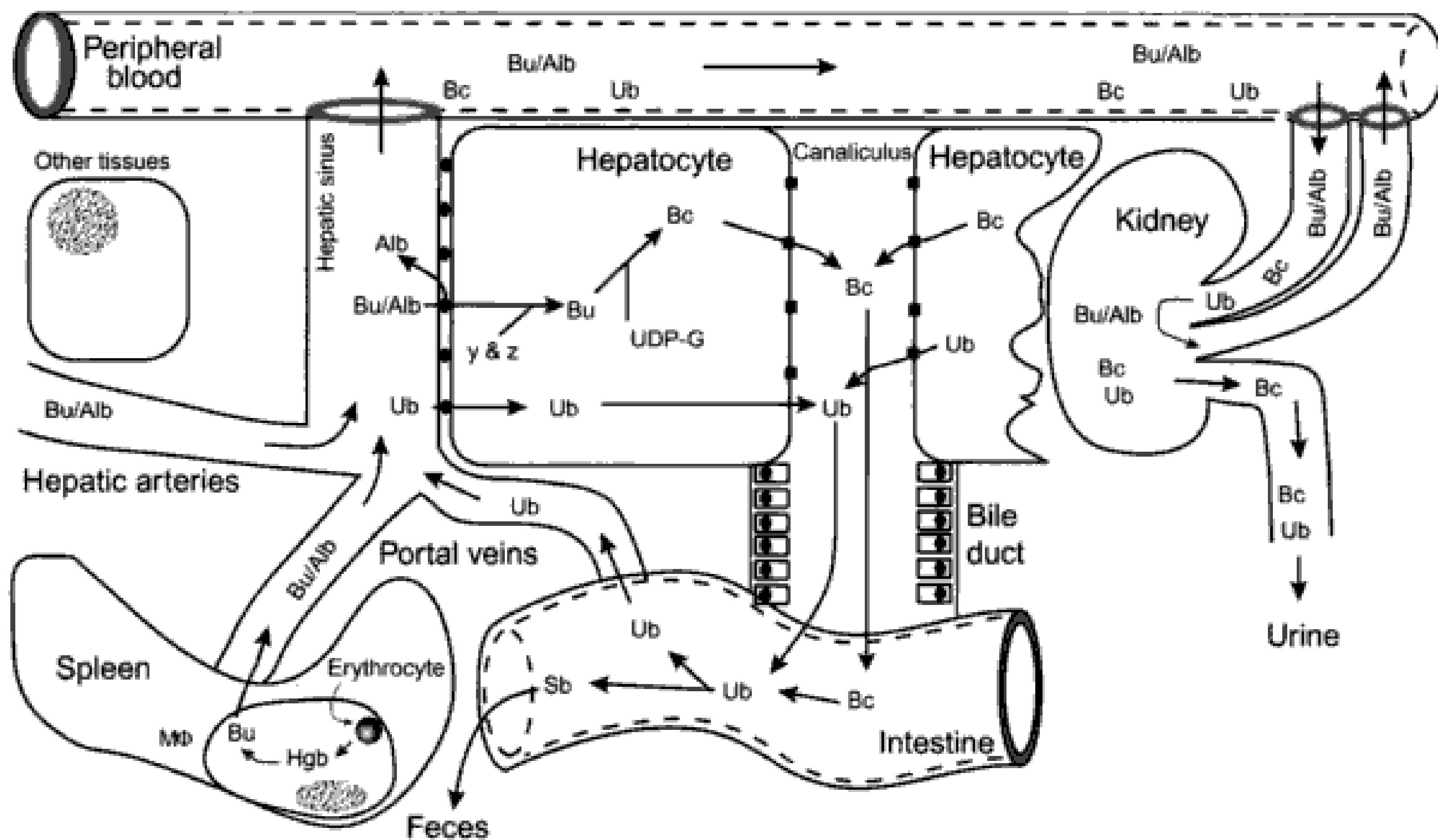

Fig. 13.1. Physiologic processes involving bilirubin. (Bu/Alb = Bu associated with albumin, Bδ-Alb = Bδ bonded to albumin, MΦ = macrophage, UDP-G = uridine diphosphoglucuronide, Sb = stercobilinogen, Ub = urobilinogen)

- In health, erythrocyte destruction within macrophages of the spleen, liver, or bone marrow results in degradation of heme to Bu. Small and usually clinically insignificant amounts of Bu are formed from heme degradation associated with ineffective erythropoiesis and degradation of other heme-containing molecules (catalase, peroxidase, cytochromes). As Bu leaves a macrophage, it forms a noncovalent association with albumin (Alb) and remains associated with albumin until uptake by hepatocytes. Bu is relatively water insoluble prior to binding to albumin.
- When Bu enters the liver and its protein-permeable sinusoids, it enters hepatocytes without albumin and binds to y-protein (ligandin) or z-protein (fatty-acid binding protein). Bu probably enters hepatocytes by a passive but facilitated process; binding proteins enhance the process by reducing the efflux of Bu back to the sinusoidal plasma.
- Within hepatocytes, Bu is conjugated with glucuronide (glucose in horses) to form bilirubin monoglucuronide or bilirubin diglucuronide, collectively called Bc.
- 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 bile acids.
- Bc in bile enters the intestine and is degraded to urobilinogen (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 (dark brown) and excreted in feces.
- If Bc escapes hepatocytes and enters blood, it can pass through the glomerular filtration barrier and be excreted in urine. Spontaneous changes in plasma Bc result in covalent binding of the modified Bc to form Bδ. Because albumin does not pass through the glomerular filtration barrier of most mammals, Bu/Alb and Bδ-Alb do not enter urine in those animals.

   b. From the measured [Bt] and [Bd], [Bi] is calculated by subtraction: [Bt] - [Bd] = [Bi].

2. If these assays are used, laboratories may report the following concentrations that should represent the following bilirubin fractions. [Bδ] is not specifically measured and cannot be calculated by these methods.

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a. [Bt] (measured) = [Bc] + [Bδ] + [Bu]
b. [Bd] (measured) = [Bc] + [Bδ]
c. [Bi] (calculated) = [Bt] - [Bd] = [Bu]

C. Dry chemical methods using thin-layer reagent slides (Kodak® or Vitros® instruments)
1. The assays use modified bilirubin diazo-reactions in thin-layer reagent films and are designed to measure the following:
a. [Bt] = [Bc] + [Bδ] + [Bu]
b. [Bu]
c. [Bc]
2. From those measured concentrations, the following are calculated.
a. [Bδ] = [Bt] - [Bc] - [Bu]
b. [Bd] = [Bc] + [Bδ]

D. Procedural notes
1. Hgb interferes with azo-reactions and produces falsely low [Bt] with Malloy-Evelyn and Jendrassik-Gróf methods. However in the thin-layer reagent slides, Hgb falsely increases the [Bt] and [Bc] and falsely decreases [Bu].
2. Light (ultraviolet) degrades bilirubin (direct sunlight: up to 50% decrease in 1 hr).
3. Using thin-layer slide assays, the sum of [Bc] and [Bu] may exceed the measured [Bt] (especially in horses). It has not been established if the [Bt] is falsely decreased or if the sum of [Bc] and [Bu] is falsely increased.

III. Hyperbilirubinemia (Table 13.4)
A. Hyperbilirubinemia occurs when the rate of Bu production exceeds the rate of Bu uptake by hepatocytes, or the rate of Bc formation in hepatocytes exceeds the rate of Bc excretion in bile. Hyperbilirubinemia may persist after removal of the cause (e.g., obstruction) because of the long half-life of Bδ.
B. Increased Bu production

Table 13.4. Diseases and conditions that cause hyperbilirubinemia

Increased Bu production
  "Hemolytic disorders, especially extravascular hemolysis (see Table 4.8)
Decreased Bu uptake by hepatocytes
  "Fasting or anorexia (especially in horses)
  Decreased functional mass (diffuse hepatocellular disease; see text for examples)
Decreased Bu conjugation
  Decreased functional mass (diffuse hepatocellular disease; see text for examples)
Decreased Bc excretion in bile
  Obstructive cholestasis
    "Hepatic cholestasis: lipidosis, lymphoma, histoplasmosis, cytauxzoonosis, cirrhosis cholangitis, cholangiohepatitis
    "Post-hepatic cholestasis: cholangitis, bile duct neoplasia, liver flukes, cholelithiasis, cholecystitis, pancreatitis, pancreatic neoplasia
  Functional cholestasis (sepsis-associated cholestasis)
Persistence of Bδ in plasma

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Depending on the assay method, hemolysis may cause either positive or negative interference with bilirubin assays.

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1. Hemolytic (prehepatic) icterus (Fig. 13.2)
   a. Disorders
      (1) Hemolytic disorders that may cause icterus are listed in Table 4.8. Animals with acute intravascular hemolytic disorders are less likely to be icteric initially.
      (2) If the rate of extravascular hemolysis is low, the hepatobiliary system may be able to eliminate Bu so it does not accumulate in plasma.
   b. Pathogenesis
      (1) 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 (Fig. 13.2).
      (2) Pathogeneses of hemolytic diseases are described in Chapter 4.
   c. Results of serum bilirubin profile (Table 13.5)
      (1) [Bt] may be mildly to markedly increased.
      (2) Initially, [Bu] >> [Bc]. If persistent, then [Bu] may approximate [Bc]. Secondary (e.g., hypoxic hepatocellular degeneration) or concurrent hepatobiliary disease may complicate the pattern.
      (3) It has been written that bilirubin fractions do not help differentiate hemolytic and hepatobiliary icterus because [Bc] is always greater than [Bu].[6] This conclusion appears to be supported by findings in a group of eight dogs that had

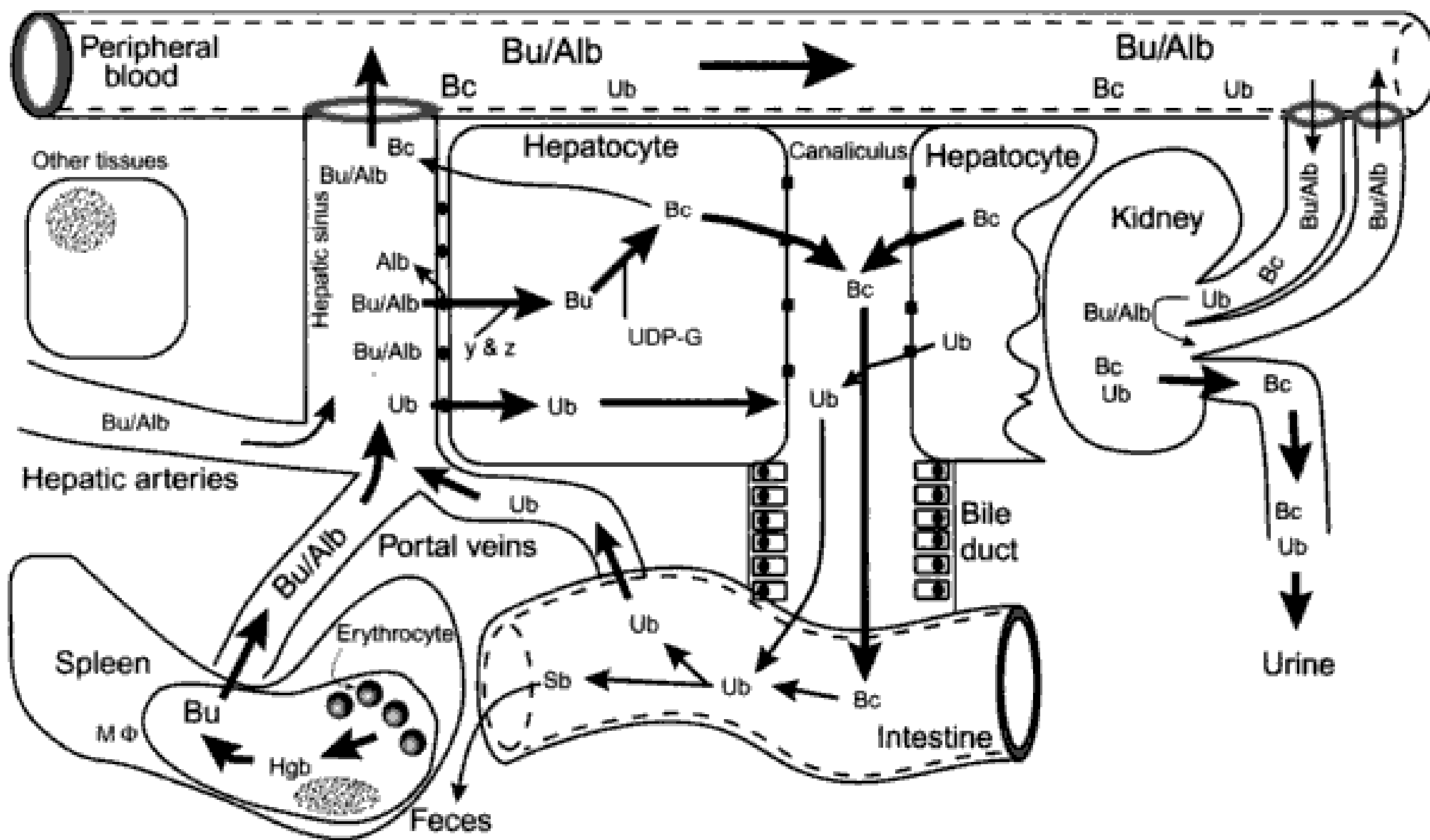

Fig. 13.2. Hemolytic icterus. (Bu/Alb = Bu associated with albumin, Bδ-Alb = Bδ bonded to albumin, MΦ = macrophage, UDP-G = uridine diphosphoglucuronide, Sb = stercobilinogen, Ub = urobilinogen)
Accelerated destruction of erythrocytes in macrophages of spleen (also liver and marrow) causes increased production and delivery of Bu to hepatocytes. If the rate of Bu formation exceeds the liver's ability to clear Bu from plasma, hyperbilirubinemia dominated by Bu will develop. Increased delivery of Bu to hepatocytes also causes increased Bc formation and biliary excretion. If Bc formation exceeds Bc transport to canaliculi, then Bc can be "regurgitated" to plasma. The increased [Bc] in plasma will result in increased Bc excretion in urine (bilirubinuria).

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(3) Lack of colostrum intake was considered a contributing factor to icterus in veal calves.[10]

b. Pathogenesis: The fasting state leads to increased mobilization of fat (lipolysis in adipocytes) and thus increased free fatty-acid concentration in blood. The free fatty acids interfere with Bu uptake by hepatocytes and thus plasma [Bu] increases.[9] The interference may be competitive because fatty acids and Bu bind to the same cytoplasmic receptor protein (z-protein or fatty-acid binding protein).[3]

c. Species comments
   (1) Horses might be more prone to this form of icterus because they conjugate bilirubin with glucose rather than glucuronic acid.[11] 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 free fatty acids.[12] It was not established if the lower bilirubin concentration was due to availability of glucose or reduction in free fatty acids.
   (2) Similar interferences in bilirubin excretion likely occur in other animals, but changes in [Bt] are minimal. Anorectic sick cats occasionally have slight hyperbilirubinemias (increased [Bu]) and thus it is easy to speculate that free fatty acids are contributing to defective Bu clearance.
   (3) Increased [Bu] may also occur in horses that are not anorectic or anemic. The pathogenesis of this hyperbilirubinemia is not understood.

d. Results of serum bilirubin profile (Table 13.5)
   (1) In horses, [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 hepatocyte uptake.

e. Other expected laboratory findings
   (1) In horses, fasting or anorexia does not produce abnormalities in other routine laboratory tests. Thus, hepatic enzymes are expected to be WRI if hepatobiliary disease is not present. Hct is expected to be WRI unless anemia is produced by the disorder that is causing the anorexia.
   (2) In cattle with hepatic lipidosis, the accumulation of lipid leads to hepatocyte swelling and damage. Thus, serum activities of hepatic enzymes may be increased.

2. Decreased functional hepatic mass
   a. A marked reduction in functional hepatocytes can result in decreased Bu uptake by hepatocytes, decreased Bu conjugation, and decreased Bc excretion. However, if a disease is causing only decreased functional mass and not obstructive cholestasis, then clinical icterus is not expected.
   b. Disorders that cause decreased functional mass are mentioned in Abnormal Results of Routine Laboratory Tests, IV.B above.

3. Hereditary deficiencies in hepatic uptake of Bu may cause hyperbilirubinemia and have been recognized in Southdown sheep[13] and Corriedale sheep.[14]

D. Decreased Bu conjugation
   1. As mentioned above, a marked reduction in functional mass would result in a reduction of Bu conjugation. When there is saturation of cytoplasmic receptor proteins, the uptake of Bu by hepatocytes decreases.
   2. Hereditary deficiencies of enzymes that catalyze conjugation reactions occur in people and laboratory animals but have not been recognized in our common domestic mammals.

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B. Because of the low renal threshold for Bc, bilirubinuria may be detected before hyperbilirubinemia is detected. If hyperbilirubinemia (from 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 small bilirubin reaction; moderate 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[19]

a. Icterus index is determined by comparing the color of a patient's plasma to a set of standard solutions containing potassium dichromate. Icterus index is recorded in units from 0 to 100. 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. Hitachi method

a. The Hitachi chemistry analyzers estimate the bilirubin concentration by spectrophotometric assessment of absorbance changes due to the presence of bilirubin. The method involves consideration of absorbance changes due to hemoglobin or lipids, if present.

b. The icteric index is reported as a numerical value (e.g., 10) that corresponds to absorbance changes expected by a [Bt] of 10 mg/dL.

3. Clinical laboratories 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 (depends somewhat on species and observer's experience).

BILE ACID (BA) CONCENTRATION[20]

I. Physiologic processes (Fig. 13.4)

A. In health, the enterohepatic circulation of bile acids is highly efficient and nearly all bile salts excreted in bile are returned to the liver via intestinal absorption and portal blood flow.

B. Bile salts are the major solids in bile that enter the intestine after gall bladder contraction. After intestinal absorption of BA, higher [BA] in portal blood may exceed the liver's ability to extract BA and thus, there is a postprandial increase in [BA] in systemic blood. Typically, gall bladder contraction results from actions of cholecystokinin that is released after ingestion of a meal. However, gall bladder contraction may occur at other times.

C. BA excretion from hepatocytes to canaliculi occurs through two processes; one is Na^+-dependent and one is not.

II. Analytical concepts

A. Terms and units

1. In common use, the term *bile acids* refers to a group of cholesterol-derived anionic acids and their dissociated anions. The bile acids include cholic acid, chenodeoxy-

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hepatocyte canalicular membranes and thus impair BA excretion.[16,17] The resultant impairment in BA excretion is called functional cholestasis (see Bilirubin Concentration, III.E.2 above).

B. Mild increases in serum [BA] occur in healthy animals after meals. Postprandial increases are also exaggerated in some hepatobiliary disorders. These findings are the basis of the bile acid challenge test (see section IV below).

C. In horses, increased serum [BA] 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 [BA] (reference interval not stated but upper limit estimated from a graph to be 18 μmol/L).[22]

D. In cattle, the diagnostic sensitivity of serum [BA] varied among liver disorders: fascioliasis (100%, $n = 11$), biliary calculi (100%, $n = 2$), hepatic abscesses (53%, $n = 15$), leptospirosis (71%, $n = 7$), hepatic lipidosis (86%, $n = 36$) (reference interval not stated but upper limit estimated from a graph to be 40 μU/L).[23] In another report, values for serum [BA] were increased in cattle with hepatic lipidosis, hepatic abscesses, leptospirosis, and fascioliasis with mean concentrations of 90–225 μmol/L at initial evaluation.[24]

IV. Bile acid challenge test for dogs and cats

A. Principle: A 12-hr fasting [BA] provides a baseline assessment of the amount of BA that escapes enterohepatic circulation and enters systemic blood. After ingestion of a standardized meal, gall bladder contraction releases bile salts to the intestine from which they are absorbed and then enter portal blood. This influx of endogenous bile salts challenges the ability of the hepatocytes to keep bile salts within the enterohepatic circulation.

B. Procedure
1. A fasting sample is collected from the dog or cat after a 12-hr fast. Then, the animal is observed while it ingests food containing protein and fat (e.g., 2 teaspoons of a canned food for those < 10 lb, 2 tablespoons for large dogs). A 2-hr postprandial sample is collected.
2. Blood collection and processing methods should not cause hemolysis because Hgb interferes with the BA assay. Postprandial lipemia should be avoided by first collecting a fasting sample and then limiting the amount of ingested food.

C. Published data
1. Dogs[25] (Table 13.7)
   a. Fasting and postprandial samples had high diagnostic sensitivity for liver disease and portosystemic shunts, but they did not consistently differentiate the type or severity of the disease. Some dogs with nonhepatic diseases may have increased fasting and postprandial [BA], but increases are typically minor.
   b. Diagnostic sensitivity and specificity (for presence or absence of liver disease) approached 100% when the following criteria were used to classify the results as positive (data from a total BA enzymatic assay).
      (1) Fasting [BA] > 20 μmol/L
      (2) 2-hr postprandial [BA] > 25 μmol/L
2. Cats[26] (Table 13.8)
   a. Fasting and postprandial [BA] had high diagnostic sensitivity for hepatobiliary disease but they did not differentiate the type or severity of the diseases.
   b. Diagnostic specificity for the absence of liver disease approached 100% when values > 20 μmol/L were considered as increased for fasting or postprandial samples, but the diagnostic sensitivity for hepatobiliary disease was only 49% (fasting) and

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Table 13.7. Results of bile acid challenge test in dogs

Group	*n*	Percentage with fasting [BA] > 5 µmol/L	Percentage with 2-hr postprandial [BA] > 15.5 µmol/L
Healthy	66	2.5[a]	2.5
Severe liver disease[b]	62	86.9	78.7
All liver disease[c]	101	82.5	77.7
Portosystemic shunt	29	100	100
Ill, liver disease not detected[d]	40	17.5[e]	12.5[f]

Source: Center SA, ManWarren T, Slater MR, Wilentz E. 1991. Evaluation of 12-hr preprandial and 2-hr postprandial serum bile acids concentrations for diagnosis of hepatobiliary disease in dogs. J Am Vet Med Assoc 199:217-226.

[a] Because the upper limit of the reference interval was determined as the value 2 standard deviations above the mean, the 2.5% assumes that reference values had Gaussian distribution.

[b] Disorders included cirrhosis, chronic hepatitis, hepatic necrosis, and cholestasis.

[c] Disorders included those for the severe liver disease groups and hepatic neoplasia, glucocorticoid hepatopathy, and passive congestion.

[d] Disorders included idiopathic epilepsy, inflammatory intestinal disease, metastatic neoplasia without hepatic involvement, disseminated intravascular coagulation, brain neoplasia, hypoadrenocorticism, glomerulonephritis, peritonitis, meningoencephalitis, endocarditis, congestive heart failure, and cystitis. Each dog lacked histologic evidence of hepatobiliary disease.

[e] None exceeding 17.1 µmol/L.

[f] None exceeding 21.9 µmol/L.

Table 13.8. Results of bile acid challenge test in cats

Group	*n*	Percentage with fasting [BA] > 5 µmol/L	Percentage with 2-hr postprandial [BA] > 10.0 µmol/L
Healthy	?	2.5[a]	2.5
All liver disease	82	73	98
Portosystemic shunt	24	79	100
Liver disease but not shunt[b]	58	71	97
Ill, liver disease not detected[c]	26	11.5[d]	30.8[e]

Source: Center SA, Erb HN, Joseph SA. 1995. Measurement of serum bile acids concentrations for diagnosis of hepatobiliary disease in cats. J Am Vet Med Assoc 207:1048-1054.

[a] Method of establishing reference interval was not reported. For the purpose of comparison, the 2.5% assumes that reference values had a Gaussian distribution.

[b] Diagnoses included hepatic lipidosis (20), hepatic necrosis (13), hepatic neoplasia (8), and cholestasis disorders (17).

[c] Diagnoses included a variety of nonhepatic disorders. Each cat lacked histologic evidence of hepatobiliary disease.

[d] None exceeding 16.0 µmol/L.

[e] Maximal value not reported.

81% (postprandial) if the same concentration was used as a cutoff point (data from a total BA enzymatic assay).

D. Factors other than hepatobiliary and portal systems that influence results
   1. Fasting [BA]
      a. Spontaneous contraction of the gallbladder may increase fasting [BA] unexpectedly.

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b. Fasting serum [BA] is dependent on the enterohepatic cycle; e.g., intestinal diseases may influence intestinal absorption of BA and thus lower a serum [BA].

2. 2-hr postprandial [BA]
   a. The peak [BA] may not occur 2 hr postprandially because of variations in gastric emptying, intestinal transit time, intestinal absorption, or other factors.
   b. The animal may not have eaten the provided food, or it may have a poor cholecystokinin response.
   c. The gall bladder may have contracted prior to feeding.
3. The above factors may contribute to the occasional finding that the fasting [BA] > 2-hr postprandial [BA]. The lower [BA] in the 2-hr postprandial sample is not of diagnostic value.

V. Conclusions
   A. The diagnostic value of a serum [BA] is primarily for detecting hepatobiliary disease or portosystemic shunts.
   B. The highest serum [BA] occurs when there is a disease process that allows BA molecules to escape enterohepatic circulation. However, many diseases or pathologic states can cause the dysfunction.
   C. The popularity of the bile acid test (compared to other liver function tests) is probably due to its simplicity; i.e., it does not require special patient preparation, sample collection, or sample handling.

AMMONIUM (NH_4^+) CONCENTRATION IN PLASMA

I. Physiologic processes (Fig. 13.5)

II. Analytical concepts
   A. Units and terms
      1. The analyte is commonly called "blood ammonia," but dominant form in plasma is ammonium. At a pH of 7.4, the NH_4^+:NH_3 ratio is about 30 and thus there is relatively little NH_3 in plasma.[1] Routine clinical assays use plasma and not whole blood as a sample.
      2. Unit conversion[5]
         a. Ammonia: µg/dL × 0.5871 = µmol/L (SI unit, nearest 5 µmol/L)
         b. Ammonium: µg/dL × 0.5543 = µmol/L (SI unit, nearest 5 µmol/L)
   B. Assays for ammonium concentration
      1. Methods
         a. Enzymatic method: NH_4^+ + α-ketoglutarate + NADPH → glutamate + NADP
         b. If an alkalinating agent is used to convert NH_4^+ to NH_3, NH_3 can be measured by a dye-binding method.
         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.[27]
   C. Samples
      1. 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, III.A.4 below).[28]

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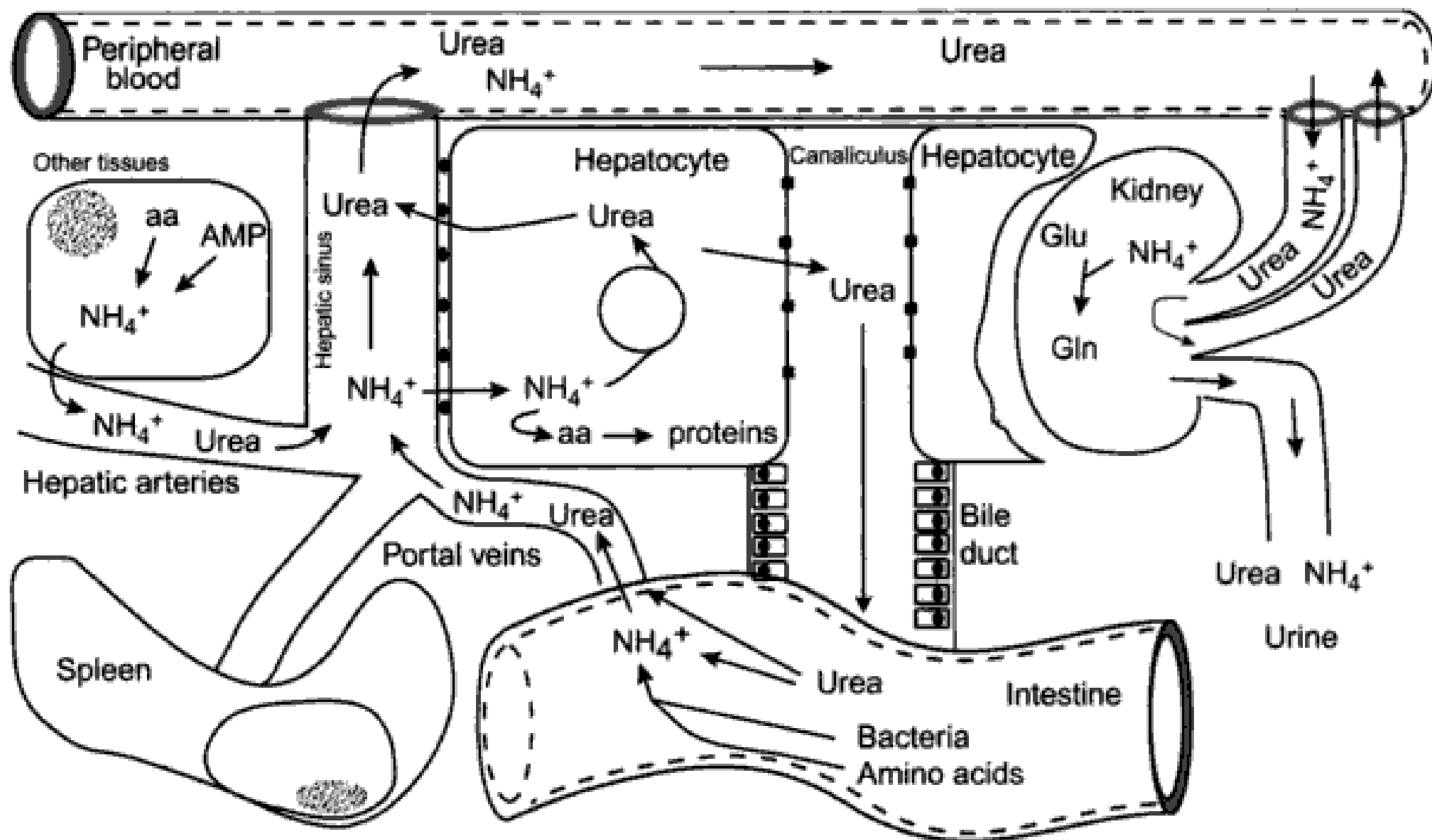

Fig. 13.5. Physiologic processes of ammonium.

- 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 AMP (especially in muscle fibers).
- After NH_4^+ enters the liver (via portal vein or hepatic artery), it enters hepatocytes and is used for the synthesis of urea (in urea cycle), amino acids, and proteins. Urea diffuses from hepatocytes to sinusoidal blood or bile canaliculi from which it may be excreted via kidneys or intestine, respectively. Urea that enters the intestine may be reabsorbed as part of an enterohepatic circulation.
- Renal excretion of NH_4^+ may occur by NH_4^+ passing through the glomerular filtration barrier and being excreted in urine. NH_4^+ is also fixed in renal tubular cells during synthesis of glutamine (Gln) from glutamate (Glu).
- 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.

2. [NH_4^+] in serum samples is greater than in paired plasma samples.[1]
3. Hemolysis causes false increases of [NH_4^+] in some assays because the heme pigment interferes with light transmittance, or because erythrocytes contain NH_4^+.[29]
4. Sample handling
    a. Ideal: Heparinized blood is delivered to a laboratory immediately after collection; plasma is harvested immediately after arrival, plasma is cooled to 4°C (in ice-bath); chemical analysis is completed within 1 hr. All steps should be completed with minimal exposure to air.
    b. Plasma [NH_4^+] is usually considered to be stable for up to 4 hr at 4°C and adequately stable for 1–2 days at -20°C.
    c. Suboptimal handling and instability problems can lead to highly variable results.[30]
        (1) Within hours of storage at room temperature, [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 allows NH_4^+ to be produced by erythrocytes and leukocytes, thus increasing measured [NH_4^+].

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Table 13.9. Diseases and conditions that cause hyperammonemia

*Decreased NH_4^+ clearance from portal blood
*Decreased functional mass: diffuse hepatocellular disease
*Decreased portal blood flow to liver: congenital and acquired portosystemic shunts
Urea cycle enzyme deficiencies (congenital)
Increased NH_4^+ production
Postprandial
Urea toxicosis in cattle
Strenuous exercise (race horses and dogs)
Urinary infection with urease-containing bacteria and concurrent urethral obstruction
Increased NH_4^+ intake
NH_4Cl administration per os or per rectum
Ammoniated forage toxicosis in cattle

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Falsely increased [NH_4^+] may occur if delayed analysis of sample results in proteolysis or [NH_4^+] production by blood cells.

(3) Delayed analysis of plasma allows $NH_{3(g)}$ to escape from plasma if the sample is not stoppered or if an evacuated tube is incompletely filled; loss of $NH_{3(g)}$ will cause a decrease in plasma [NH_4^+].

III. Hyperammonemia (Table 13.9)
   A. Decreased clearance of NH_4^+ from portal blood
      1. Diseases or conditions that reduce the removal of NH_4^+ from portal blood may result in 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, lipidosis
         b. Portosystemic shunts, either congenital or acquired
      2. Decreased NH_4^+ clearance can potentially be due to congenital deficiencies involving the urea cycle (rare disorders).
         a. Argininosuccinate synthetase deficiency has been reported in dogs.[31]
         b. Defective mitochondrial transporter of ornithine, a substance that is transported from cytosol to mitochondria in the urea cycle, has caused hyperammonemia, hyperornithinemia, and homocitrullinuria syndrome in Morgan fillies.[32]
      3. The [NH_4^+] of Irish wolfhound puppies that were 7–8 weeks old was 47–115 µmol/L compared to 6–27 µmol/L for adults. The pathogenesis of the hyperammonemia in these pups was not determined, but Irish wolfhounds do have a high incidence of portosystemic shunts.[33]
      4. In 69 dogs with hepatic encephalopathy due to several liver diseases (including 14 congenital and 29 acquired shunts), [NH_4^+] in arterial plasma averaged 1.5 times the [NH_4^+] of venous plasma. The venous concentrations may be lower because of enhanced NH_4^+ clearance from arterial blood by kidneys, muscle, or other tissues. Because of the difference, some investigators recommend that arterial samples should be collected for plasma [NH_4^+].[28] 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.

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B. Increased NH_4^+ production

1. Postprandial: Increased NH_4^+ production during the digestion of a meal may result in temporary hyperammonemia. This condition should not be considered if the animal was appropriately fasted prior to sample collection.
2. Urea toxicosis in cattle: Hydrolysis of urea in the rumen liberates NH_3 that combines with H^+ to form NH_4^+. This makes ruminal fluid more alkaline. 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 plasma [NH_4^+].[34]
3. Strenuous exercise: During exercise, two ADP molecules combine to form AMP and ATP via the myokinase reaction of myocytes. The deamination of AMP produces inosine monophosphate and NH_3. The NH_3 diffuses to blood and increases plasma [NH_4^+] and pH.[35]
   a. In greyhounds, plasma [NH_4^+] increased from a mean of 82 µmol/L pre-race to a mean of 256 µmol/L immediately after the run.[36]
   b. In quarter horses, plasma [NH_4^+] increased from a mean of 67 µmol/L pre-exercise (treadmill) to a mean of 137 µmol/L immediately after exercise.[37] In another study, the mean [NH_4^+] pre- and post-exercise was 37 and 113 µmol/L, respectively.[35]
4. 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 resulted from increased NH_4^+ production in the urinary tract, decreased renal excretion due to obstruction, and perhaps decreased urea cycle activity due to acidosis.[38]

C. Increased NH_4^+ intake

1. NH_4Cl administration per os or via colon (see NH_4^+ Tolerance Test below)
2. Ammoniated forage toxicosis in calves: A 30-day-old calf had a plasma [NH_4^+] that was considered increased while nursing a cow that had ingested hay treated with anhydrous ammonia.[39]

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 portal veins. Either decreased hepatic functional mass or a portosystemic shunt will allow NH_4^+ to escape the enterohepatic circulation and cause an excessive increase in 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. Typically, an NH_4^+ tolerance test is not indicated if there is a 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-hr fast and collection of a fasting sample, NH_4Cl is given orally (0.1 g/kg but not more than 3 g) in 20–50 mL H_2O. A 30-min post-NH_4Cl blood sample is collected.
2. *Rectal NH_4^+ tolerance test:* After a 12-hr fast and collection of a fasting sample, NH_4Cl is given (5% solution; 2 ml/kg) via a catheter inserted 20–35 cm into the colon. Blood samples are collected 20 and 40 min later. Both 20-min and 40-min samples have been recommended because peak [NH_4^+] occurred before 30 min in some dogs and after 30 min in other dogs.[40]

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Table 13.10. Ammonium tolerance test results for 6 dogs with portosystemic shunts

Results for Assay #1	10 healthy dogs[a]	Dog 1[b]	Dog 2[c]	Dog 3[c]
Fasting [NH_4^+] (µg/dL)	88 ± 36	100	370	260
30-min [NH_4^+] (µg/dL)	155 ± 71	1000	1400	850

Results for Assay #2	10 healthy dogs[a]	Dog 4[d]	Dog 5[c]	Dog 6[c]
Fasting [NH_4^+] (µg/dL)	56 ± 14	100	250	125
30-min [NH_4^+] (µg/dL)	76 ± 30	550	900	650

Source: Meyer DJ, Strombeck DR, Stone EA, Zenoble RD, Buss DD. 1978. Ammonia tolerance test in clinically normal dogs and in dogs with portosystemic shunts. J Am Vet Med Assoc 173:377-379.

Note: Two sets of data reported because of lack of analytical agreement of the two ammonium assays (note different values in healthy dog groups).

[a] All data for healthy dogs expressed as mean ± standard deviation.

[b] Dog 1: Fasting [NH_4^+] was WRI and thus hepatic function was sufficient to handle the NH_4^+ load during the fasting state. The marked hyperammonemia in the 30-min sample is consistent with a portosystemic shunt or hepatic insufficiency.

[c] Dogs 2, 3, 5, and 6: The fasting hyperammonemia revealed the defective ammonium clearance; the marked hyperammonemia in the 30-min sample would be expected because of influx of NH_4^+ into the system.

[d] Dog 4: Fasting [NH_4^+] was slightly increased and thus not strong evidence of hepatic insufficiency or a portosystemic shunt. However, [NH_4^+] in 30-min sample was definitely increased and thus supportive of hepatic insufficiency or a portosystemic shunt.

D. Interpretation of results
   1. Fasting [NH_4^+]
      a. WRI: 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; no evidence of a rare congenital urea cycle enzyme deficiency
      b. Hyperammonemia: See Table 13.9 for potential pathogeneses.
   2. Postchallenge [NH_4^+]
      a. WRI: no evidence of hepatic insufficiency, portosystemic shunt, or a rare congenital urea cycle enzyme deficiency
      b. Increased above reference interval: decreased functional hepatic mass, portosystemic shunt, or both; rarely, a congenital urea cycle enzyme deficiency
E. Example results for dogs with portosystemic shunts are in Table 13.10.

DYE EXCRETION TESTS

Prior to availability of spectrophotometric assays for bile acids, hepatic function was clinically assessed by excretion tests involving two organic anions or dyes: BSP (bromosulfophthalein or sulfobromophthalein) and ICG (indocyanine green).

Excretion of these dyes is dependent upon hepatic blood flow, hepatocyte uptake, conjugation (BSP but not ICG), excretion to the biliary system, and enterohepatic circulation (BSP but not ICG). 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 [BA] and [Bt] provides essentially the same information about hepatic function.

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Chapter 14

GLUCOSE AND RELATED REGULATORY HORMONES

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Table 14.1. Abbreviations and symbols in Chapter 14

»	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
ACTH	adrenocorticotrophic hormone (corticotropin)
BVD	bovine virus diarrhea
DM	diabetes mellitus
ELISA	enzyme-linked immunosorbent assay
GH	growth hormone
GHRH	growth hormone releasing–hormone
GLP	glucagon-like peptide
GLUT	facilitative glucose transporter
Hct	hematocrit
IRG	immunoreactive glucagon
IRI	immunoreactive insulin
IRI:G	immunoreactive insulin to glucose
NADH	reduced nicotinamide adenine dinucleotide
NADH:NAD	reduced to oxidized nicotinamide adenine dinucleotide
NaF	sodium fluoride
OGTT	oral glucose tolerance test
RIA	radioimmunoassay
SI unit	Système International d'Unités
UN	urea nitrogen
WRI	within reference interval

GLUCOSE CONCENTRATION IN SERUM, PLASMA, OR WHOLE BLOOD

I. Physiologic processes (Fig. 14.1)
   A. Blood glucose concentrations are regulated and influenced by several hormones. For the hormones to influence metabolism, there must be appropriate receptors and transport systems in the target cells.
      1. Insulin activity lowers blood glucose concentrations by promoting the uptake, utilization, or storage of glucose by hepatocytes, myocytes, and adipocytes. Insulin is not needed for glucose transport into neurons, leukocytes, erythrocytes, 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. Glucose entry into most cells is modulated by a family of proteins called facilitative glucose transporters (GLUT-1 to GLUT-7). Insulin promotes glucose entry into myocytes and adipocytes via GLUT-4; insulin is not needed for the other carriers (e.g., GLUT-2 in hepatocytes).
      3. Glucagon activity increases blood glucose concentrations by stimulating gluconeogenesis and glycogenolysis.
      4. Catecholamine activity alters blood glucose concentrations by several mechanisms.
         a. An α_2-adrenergic stimulus of pancreatic β-cells decreases insulin release and thus reduces glucose utilization by hepatocytes, myocytes, and adipocytes.
         b. A β-adrenergic stimulus of pancreatic β-cells increases insulin release.
         c. A β_2-adrenergic stimulus of hepatocytes increases glycogenolysis.
         d. An α_1-adrenergic stimulus of the pituitary gland increases GH release.

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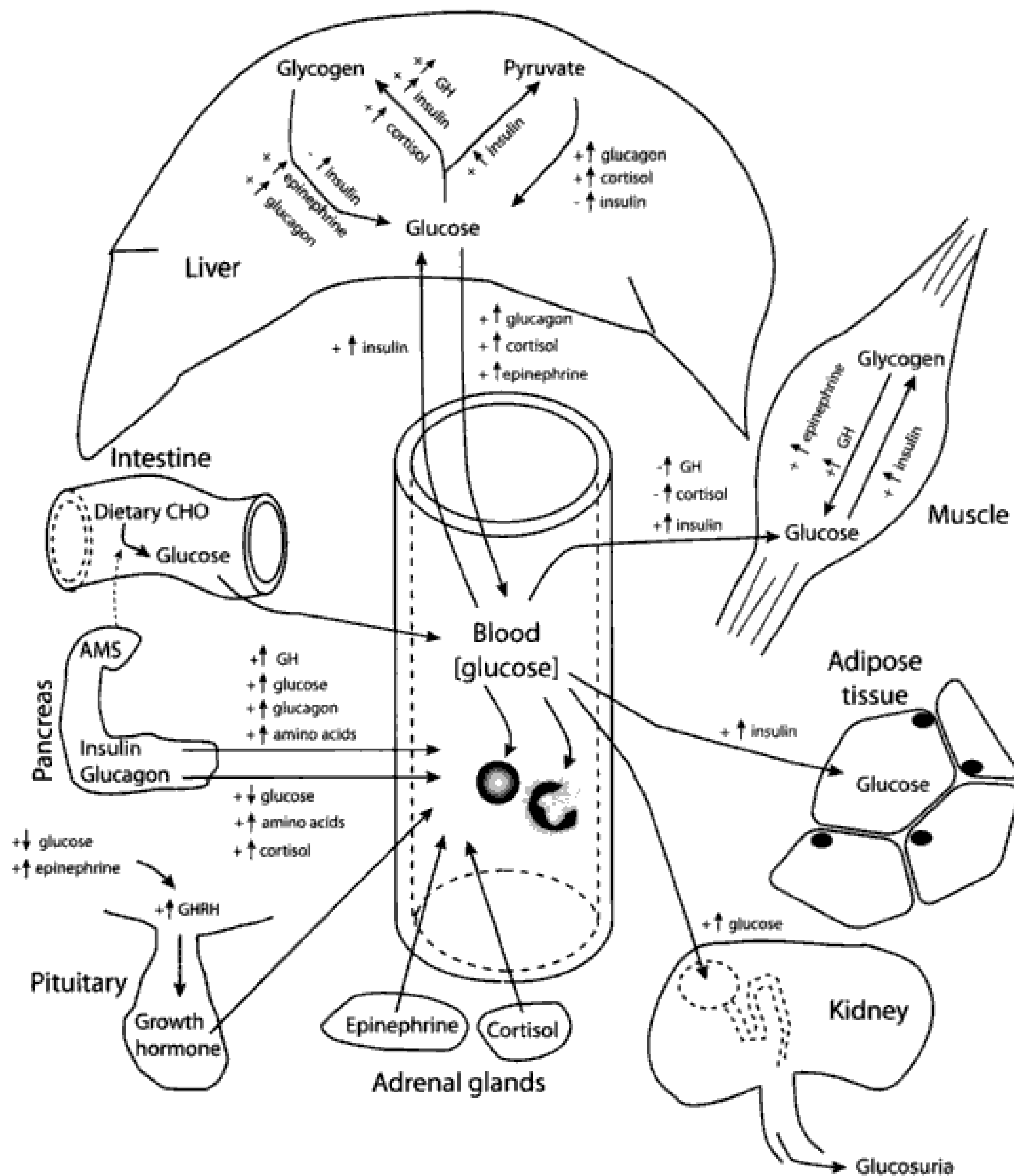

Fig. 14.1. Physiologic factors that influence blood glucose concentration.

- *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 they are not removed by hepatocytes.
- *Pancreas:* Insulin and glucagon are released from pancreatic islet cells—β-cells and α-cells, respectively. Insulin secretion is stimulated by increased blood concentrations of glucose, GH, glucagon, or amino acids. Glucagon secretion is stimulated by increased blood concentrations of amino acids and cortisol, or by decreased blood glucose concentrations.
- *Liver:* Hepatocytes are the primary source of blood glucose during fasting. Glucose can be obtained from glycogenolysis (stimulated by epinephrine and glucagon but inhibited by insulin) or gluconeogenesis (stimulated by glucagon and cortisol but inhibited by insulin). Insulin also promotes glycolysis. Increased glucose release from hepatocytes is promoted by increased glucagon, cortisol, or epinephrine. Insulin promotes the uptake of glucose by promoting glucokinase activity.
- *Muscle:* Glucose uptake by myocytes is promoted by insulin through specific insulin receptors and glucose transporters; GH and cortisol inhibit the uptake of glucose. Insulin promotes glycogen synthesis in myocytes whereas GH and epinephrine promote glycogenolysis in muscle (perhaps only cardiac muscle).
- *Adipose tissue:* Insulin promotes the uptake of glucose by adipocytes.
- *Kidney:* If the renal threshold is exceeded, then hyperglycemic glucosuria will develop.
- *Pituitary:* GH release from the pituitary is stimulated by GHRH, which is released from the hypothalamus during hypoglycemia or after epinephrine stimulation.
- *Blood cells:* Glucose enters erythrocytes, leukocytes, and platelets through insulin-independent processes and is used in glycolysis and the hexose monophosphate shunt.

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5. GH (somatotropin) activity increases blood glucose concentrations by reducing glucose uptake by myocytes and adipocytes.
6. Cortisol activity increases blood glucose concentrations by stimulating gluconeogenesis and creating a state of insulin resistance (see III.B.1.c below).

B. In monogastric animals, fasting blood glucose concentrations are maintained by gluconeogenesis using products of protein and lipid catabolism. In ruminants, propionate from rumen fermentation is used for hepatic gluconeogenesis. In the horse, colonic propionate contributes to gluconeogenesis.[1]

C. Fasting normoglycemia represents a balance between the actions of insulin (promoting storage and utilization of fuels) and glucagon (promoting mobilization of fuels). When there is an imbalance and fuel mobilization dominates, then the animal will be hyperglycemic and potentially glucosuric, which reduces the magnitude of the hyperglycemia. If fuel storage and utilization are dominant, then the animal will be hypoglycemic. Even though the balance of insulin and glucagon actions is assessed by a blood glucose concentration, these hormones also influence lipid and protein metabolism.

II. Analytical concepts

A. Terms and units
1. As explained below, the concentration of 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, serum and plasma glucose concentrations will be nearly equal to each other.
2. Unit conversion: mg/dL × 0.05551 = mmol/L (SI unit, nearest 0.1 mmol/L)[2]

B. Sample for glucose concentrations
1. For most clinical laboratory methods, serum is recommended and plasma (especially heparinized) can be used. Serum and plasma should be removed from cells within 1 hr of blood collection because glycolysis continues in blood cells *in vitro*, thus lowering glucose concentration. If plasma or serum has contact with cells at room temperature in uncentrifuged blood, glucose concentrations typically decrease about 5%–10% per hr; marked leukocytosis and erythrocytosis will accelerate the process. Special collection tubes containing NaF can be used to block glycolysis (inhibits enolase), but they are not routinely used in clinical medicine. NaF will also inhibit glucose oxidase activity (and other enzymes) and thus NaF plasma should not be used in glucose assays that use glucose oxidase.
2. Glucose concentrations in arterial and capillary blood are greater than in venous blood because peripheral tissues consume glucose; the difference in normoglycemic and normoinsulinemic states is probably < 10 mg/dL.

C. What is measured: whole blood or plasma glucose concentration?
1. When an assay uses whole blood for a sample, it is important to know if the result represents a whole blood glucose concentration, a plasma glucose concentration, or a calculated plasma glucose concentration. Some instruments measure and report whole blood glucose concentrations. Other instruments measure a whole blood glucose concentration and calculate a plasma glucose concentration. Of these, some assume a normal Hct value and thus the calculated value will not be correct in anemic or erythrocytotic samples.[3] Some whole blood assays measure molality and not molarity, and thus variations in the H_2O content of blood (e.g., different protein concentrations) will influence measured glucose concentrations.[4,5]
2. There have been several reports of comparisons of glucose concentrations measured

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Table 14.3. Criteria for the diagnosis of DM in people

1. Symptoms of diabetes plus casual plasma glucose concentration ≥ 200 mg/dL (11.1 mmol/L).[a] Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss.
   Or
2. Fasting plasma glucose ≥ 126 mg/dL (7.0 mmol/L).[b] Fasting is defined as no caloric intake for at least 8 hr.
   Or
3. 2-hr plasma glucose ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT).[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 H_2O.

In the absence of unequivocal hyperglycemia with acute metabolic decompensation, these criteria should be confirmed by repeat testing on a different day. The third measure (OGTT) is not recommended for routine clinical use.

Sources: WHO Study Group. 1985. Diabetes mellitus. In: WHO Technical Report Series, Geneva, 1-113; Gavin JR, III. 2000. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 23:S4-S19.

[a] The > 200 mg/dL criterion should work for dogs and horses; > 250 mg/dL is proposed for cats, and > 150 mg/dL is proposed for cattle.

[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, arterial disease). Until similar studies are done in domestic species, similar decision limits cannot be established for them.

[c] It is unlikely that veterinarians will need such a criterion in clinical medicine

III. Hyperglycemia
   A. As summarized above, plasma glucose concentrations are influenced by many factors and thus it should not be surprising that there are many causes of hyperglycemia. 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.[6]
      1. Definition of DM: "Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both."
      2. Diagnostic criteria for human DM (Table 14.3)[6]
         a. To adapt these criteria to domestic mammals, veterinarians will need to establish appropriate decision values for glucose concentrations in each animal species (see Table 14.3 for suggested decision values for dogs, cats, horses, and cattle).
         b. To use the criteria, veterinarians will need to expand the definition of DM commonly used in veterinary medicine. For example, hyperadrenocorticism and acute pancreatitis were reported to be "concurrent disorders" in dogs with DM rather than potential types of DM.[7]
      3. The expert committee recommended that the classifications of "insulin-dependent" and "non-insulin-dependent" DM be dropped because of the confusion generated by their use.
   B. Disorders and conditions (Table 14.4)
      1. Physiologic hyperglycemia
         a. Postprandial hyperglycemia: Glucose absorbed after carbohydrate (starch) digestion increases glucose entry into blood; also, release of glucagon promotes gluconeogenesis. Glucose concentrations should return to fasting values within 4 hr.

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superficial necrolytic dermatitis. The pathogenesis of the hyperglycemia is not established but may involve insulin resistance, glucagon, growth hormone, or alterations in amino acid, fatty acid, or zinc metabolism. It appears that the diabetic state develops after the onset of hepatic disease but it is not established if the liver disease causes the diabetic state.

(3) Drug-induced DM: persistent hyperglycemia (2 or more days) associated with use of a drug
  (a) Glucocorticoids (see III.B.1.c above)
  (b) Thyroid hormones: Studies in cats suggest that hyperthyroidism creates a state of insulin resistance; the mechanism is not known.[17,18] In a study in which thyroxine was given to create experimental hyperthyroidism in dogs, data indicated that the glucose-induced hyperglycemia was prolonged because of defective insulin secretion.[24]
  (c) Megestrol acetate: As a steroid, it promotes gluconeogenesis; as a progestin, it stimulates release of GH (see III.B.1.d above). Both mechanisms apply to dogs, perhaps only the latter in cats.[25]

(4) Infectious DM
  (a) Cattle infected with BVD virus can develop DM that appears to result from damage to β-cells.[26,27]
  (b) Sepsis: An early response to endotoxemia is insulin resistance and resultant hyperglycemia. Hypoglycemia may develop later. There are several hormonal and cellular responses in sepsis that alter glucose metabolism.[28] As animals with DM are considered more susceptible to infections (especially urinary), sepsis could be the cause or result of DM.

(5) Genetic DM: An inherited form of DM occurred in Keeshond dogs; the onset of the disorder was frequently before 6 months of age and was due to β-cell hypoplasia.[29]

(6) Uncommon forms of immune-mediated DM: anti-insulin antibodies[30]

3. Pharmacologic hyperglycemia: hyperglycemia associated with occasional or sporadic administration of a drug (persistent use of some agents may produce a disorder that fulfills diagnostic criteria for DM; see Table 14.3).
  a. Glucose-related
    (1) Oral or intravenous glucose (dextrose): Glucose enters plasma faster than it is utilized, stored, or excreted.
    (2) Glucocorticoids (see III.B.1.c above)
    (3) Megestrol acetate: As a steroid, it promotes gluconeogenesis; as a progestin, it stimulates release of GH (see III.B.1.d above). Both mechanisms apply to dogs, perhaps only the latter in cats.[25]
    (4) Ketamine:[31] Ketamine stimulates release of epinephrine, which promotes glycogenolysis and hyperglycemia.
    (5) Glucagon: Excess glucagon antagonizes insulin activity by stimulating gluconeogenesis and inhibiting glucose utilization and storage.
    (6) Thyroxine: In a study in which thyroxine was given to create experimental hyperthyroidism in dogs, data indicated that the glucose-induced hyperglycemia was prolonged because of defective insulin secretion.[24]
    (7) Ethylene glycol:[32] Ethylene glycol may inhibit glycolysis and the Kreb's cycle, and thus may indirectly stimulate gluconeogenesis in "starved" cells.
  b. Insulin-related

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Table 14.5. Diseases and conditions that cause hypoglycemia

- Pathologic hypoglycemia
  - [a]Increased insulin secretion: pancreatic β-cell neoplasia (insulinoma)
  - Decreased insulin antagonists
    - [a]Hypoadrenocorticism (decreased cortisol)
    - Growth hormone deficiency
    - Hypopituitarism (decreased cortisol and GH)
  - Decreased gluconeogenesis
    - [a]Hepatic insufficiency/failure: acquired, congenital
    - [a]Hypoadrenocorticism (decreased cortisol)
    - Neonatal or juvenile hypoglycemia
    - Starvation and severe malnutrition
  - Decreased glycogenolysis
    - Glycogen storage diseases (rare)
  - Increased glucose utilization
    - Lactational hypoglycemia (spontaneous bovine ketosis)
    - Exertional hypoglycemia (hunting dogs, endurance horses)
    - Leukocytosis, extreme
    - Erythrocytosis, extreme
  - Other pathologic hypoglycemias with uncertain or unknown pathogeneses
    - Hypoglycemia associated with non-β-cell neoplasms: epithelial and nonepithelial
    - [a]Sepsis, especially with endotoxemia
    - Pregnancy hypoglycemia
    - Chronic renal failure in a cat
- Pharmacologic hypoglycemia
  - Insulin
  - Sulfonylurea compounds (glipizide, glyburide)
  - Ethanol

Note: Delayed analysis of blood samples or failure to remove serum or plasma from blood cells appropriately will result in falsely low glucose concentrations. Bromide ions will cause falsely low glucose concentrations using the i-STAT® instrument. Whole blood glucose concentrations are lower than serum or plasma glucose concentrations and thus appropriate reference intervals should be used to determine if hypoglycemic (see text).

(1) Hepatic insufficiency: With marked reduction in functional hepatic mass due to congenital or acquired diseases, there are too few hepatocytes to maintain fasting normoglycemia. Other evidence of hepatic disease or insufficiency (e.g., increased hepatic enzyme activities, hypoalbuminemia, decreased [UN, increased bile acid concentration]) is expected.

(2) Hypoadrenocorticism (see Hypoadrenocorticism four paragraphs above).

(3) Neonatal or juvenile hypoglycemia: This neonatal canine disorder occurs in toy and miniature breeds and may be due to hepatic immaturity and insufficient gluconeogenesis relative to metabolic rate and glucose consumption.[38]

(4) Starvation: With chronic depletion of body fuels (including proteins and fats), gluconeogenesis may not be able to maintain normoglycemia. A starved state may be caused by lack of food intake, maldigestion, or intestinal malabsorption. Generally, physiologic pathways will attempt to maintain blood glu-

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cose at the expense of other body fuels, and thus animals with this form of hypoglycemia are expected to be markedly underweight or emaciated.

d. Decreased glycogenolysis: Congenital deficiencies of enzymes needed for glycogenolysis may result in hypoglycemia and accumulation of glycogen in cells (glycogen storage diseases).[39]

e. 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 will become hypoglycemic if gluconeogenesis cannot meet demand. Ketosis develops secondarily because of enhanced fatty acid catabolism.[40]
   (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. On the other hand, catecholamine and catecholamine-independent factors tend to cause hyperglycemia in exercised animals.
   (3) Leukocytosis: Extreme leukocytosis (as seen in some leukemias) has been reported to cause hypoglycemia because of increased glycolysis. Depending on sample handling, the hypoglycemia could be partially due to *in vitro* glycolysis. Increased glucose consumption may occur because of increased numbers of leukocytes or because of increased metabolic activity in leukocytes (e.g., after stimulation by granulocyte-colony stimulating hormone[41] or malignant blast cells).
   (4) Erythrocytosis (same concepts as extreme leukocytosis): A leukocyte needs more glucose than an erythrocyte but typically there are about 1000 times as many erythrocytes as leukocytes. Marked rubricytosis would probably cause increased glucose consumption.[42]

f. Other pathologic states causing hypoglycemia through unknown mechanisms
   (1) Hypoglycemia associated with non-β-cell neoplasia: Several non-β-cell neoplasms have been associated with hypoglycemia; most were leiomyomas, leiomyosarcomas, or hepatocellular carcinomas.[43-47] The hypoglycemia might result from secretion of an insulin-like substance, excessive glucose utilization by neoplastic cells, liver dysfunction, or a combination of factors.
   (2) Sepsis: Hypoglycemia is probably due to increased utilization by tissue and decreased glucose production. It is not due to glucose consumption by organisms. Endotoxins have been shown to produce hypoglycemia, possibly by increasing glucose utilization.[48]
   (3) Pregnancy hypoglycemia: A ketotic hypoglycemia occurs in late pregnancy in dogs; the pathogenesis of the disorder is not established.[49]
   (4) Chronic renal failure in a cat: The cause of the hypoglycemia was not determined and may not have been directly related to decreased renal function.[50]

2. Pharmacologic 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 a diabetic needs will depend on food intake, physical activity, and other factors.

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(2) Surreptitious insulin injections: Malicious administration of insulin to a horse has been reported.[51]

b. Sulfonylurea compounds (glipizide, glyburide): These drugs, sometimes referred to as oral hypoglycemic agents, directly stimulate insulin secretion and may also improve cellular responses to insulin.[52]

c. Ethanol: Ethanol oxidation results in generation of NADH. When there is an acute excess of ethanol, an increased NADH:NAD ratio blocks gluconeogenesis and results in hypoglycemia.[53]

IMMUNOREACTIVE INSULIN (IRI) CONCENTRATION IN SERUM OR PLASMA

I. Physiologic processes

A. Insulin is a polypeptide hormone ($M_r \approx 6000$) with 51 amino acids in two chains (A and B) linked by disulfide bridges. The amino acid sequences of insulin molecules of dogs and pigs are identical and have one amino acid difference from human insulin. Equine insulin has one amino acid difference from porcine insulin. Feline and bovine insulin molecules are similar but have minor differences from canine, porcine, and human insulin.[52,54]

B. Stimuli for insulin secretion include increased concentrations of glucose, amino acids, and several hormones (glucagon, gastrin, secretin, pancreozymin, gastrointestinal polypeptide, and β-adrenergic hormones).

C. Inhibitors of insulin secretion include somatostatin, α_2-adrenergic agonists, and β-adrenergic antagonists.[35]

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 Ca^{2+}-regulated) break peptide bonds to form insulin, C-peptide, and split peptides. Glucose concentrations regulate synthesis of proinsulin and one cleavage enzyme. Insulin and C-peptide secretions are equimolar; small amounts of proinsulin and split peptides are also released.[34]

E. The major actions of insulin are illustrated in Fig. 14.1.

II. Analytical concepts

A. Terms and units

1. IRI is the preferred term for immunoassay measurements of serum or plasma insulin for two reasons: (1) measurements may include proinsulin; (2) measurements of insulin are in immunoreactive units, not biologic activity units of injectable insulin.

2. Units: μU/mL = mU/L; μU/mL × 7.175 = pmol/L; μg/L × 172.2 = pmol/L (SI unit, nearest 5 pmol/L)[2]

B. Assays

1. Most measurements of IRI are made using commercial RIA kits and therefore use anti-insulin antibody reagents. A few ELISA assays are available.

2. The antibody in commercial assays may be an antiporcine or antihuman insulin antibody. There is sufficient cross-immunoreactivity that commercial assays have been validated for canine insulin. Commercial assays may not be valid for feline insulin.[55]

3. Wide ranges of [IRI] have been reported for canine, feline, and human samples assessed with commercial insulin assays.[34,55,56] The variation may partially be due to differences in standard or calibrator solutions, but unacceptable variation persisted after laboratories used a common calibrator.[34] Because of this variation:

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a. A patient's [IRI] should be compared against reference intervals 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 assay.
b. Laboratories offering quantitation of [IRI] should thoroughly evaluate their insulin assays for performance characteristics with varying lots of reagents.

4. Samples
   a. [IRI] may be determined in serum or heparinized plasma; EDTA-plasma can give falsely increased values in some assays.[34]
   b. IRI is stable in whole blood at room temperature for at least 5 hr. In serum, it is stable for 7 days at 4°C and for several months at -20°C. Thawing and refreezing should be avoided.[34]
   c. Because glucose, amino acids, and several gastric, intestinal, and pancreatic hormones influence insulin secretion, it is very important that samples be collected from fasted animals to reduce the effects of these physiologic factors.

III. Hyperinsulinemia
   A. The major reason for measuring [IRI] is to document the inappropriate release of insulin from neoplastic β-cells, i.e., too much insulin released for the animal's plasma or serum glucose concentration.
   B. Disorders and pathogeneses (Table 14.6)
      1. Increased insulin production and release
         a. Functional pancreatic β-cell neoplasia (insulinoma): Neoplastic β-cells may consistently or sporadically produce insulin, which results in hypoglycemia because of enhanced glycolysis, reduced gluconeogenesis, and increased glucose uptake by myocytes and adipocytes.
         b. Hyperglycemia not due to decreased insulin production
            (1) Hyperinsulinemia is expected in a physiologic hyperglycemia because hyperglycemia stimulates the production and release of insulin.
            (2) Hyperinsulinemia is expected in 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.
      2. Anti-insulin antibodies: The presence of anti-insulin antibodies may result in falsely increased [IRI] in some assays (see Fig. 17.2 for concept). Anti-insulin antibodies may result from spontaneous pathologic processes or insulin therapy.

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. However, measuring [IRI] is not common in hyperglycemic animals. The

Table 14.6. Diseases and conditions that cause hyperinsulinemia

Increased insulin production and release
Functional pancreatic β-cell neoplasia (insulinoma)
Hyperglycemic disorders not due to decreased insulin production (see text)

Note: Anti-insulin antibodies may produce a positive interference in some assays.

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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.

B. Disorders or conditions where [IRI] is expected to decrease
   1. Pathologic hypoinsulinemia
      a. Type 1 DM: decreased insulin production because of destruction of β-cells
      b. Type 2 DM: Advanced stages of pancreatic amyloidosis involve β-cell damage and thus decreased insulin production.
   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 result in falsely decreased values.

V. Immunoreactive insulin:glucose (IRI:G) ratio

A. Because insulin production and release from β-cells are dependent on plasma glucose concentrations, an IRI:G ratio should indicate if the measured [IRI] is appropriate for the degree of glucose stimulation. When used, the ratio is typically calculated using conventional units for both IRI and glucose concentrations (Eq. 14.2.).

$$\text{IRI:G} = \frac{[\text{IRI}] \times 100}{[\text{glucose}]} \qquad \textbf{(14.2)}$$

with [IRI] in μU/mL and glucose in mg/dL; thus ratio unit is μU IRI/mg glucose

B. The lack of analytical agreement between insulin assays requires that reference intervals for IRI:G ratios be established for each assay, a task that is not commonly accomplished.

C. Interpretation of fasting IRI:G ratio (assuming valid concentrations of IRI and glucose and comparison to appropriate reference interval for IRI:G ratio)
   1. Increased
      a. If associated with hypoglycemia, then insulin is contributing to the hypoglycemia.
      b. If associated with normoglycemia or hyperglycemia, then increased IRI:G ratio may indicate insulin resistance.
   2. WRI
      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: If associated with hyperglycemia, then an absolute insulin deficiency is present and could be due to β-cell damage or glucose toxicosis.[14]

D. IRI:G ratio may be difficult to interpret for reasons other than the variations created by different assays.
   1. Substances other than glucose 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 peripheral blood.

E. Amended insulin:glucose ratio 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"[57] and referenced an "in press" article by Turner, Oakley, and Nabarro. Based on the

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observation, they proposed that glucose concentrations above 30 mg/dL would result in insulin entrance into peripheral blood. To evaluate [IRI] in hypoglycemic people, they modified the IRI:G ratio by subtracting 30 mg/dL from the measured glucose concentration. If the modification was valid, there would be a direct relationship between insulin and glucose concentrations over 30 mg/dL, i.e., "x" µU of insulin for every "y" mg of glucose > 30.

2. In 1973, Turner, Oakley, and Nabarro reported changes in plasma insulin concentrations during ethanol-induced hypoglycemia in obese and nonobese people.[58]
   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 glucose concentrations of 40–65 mg/dL. There were only three samples with glucose concentrations < 30 mg/dL; insulin values in those samples ranged from 0 to 2.0 µU/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 peripheral blood. 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; a technique generally limited to experimental investigations.
3. Many veterinary publications have included the use of amended IRI:G ratio to evaluate [IRI] and glucose concentrations in domestic and nondomestic animals. There have also been several attempts to squelch the use of amended IRI:G ratio, but it seems to have a life of its own.[59-63] The amended IRI:G ratio should not be used.

IMMUNOREACTIVE GLUCAGON (IRG) CONCENTRATION IN PLASMA

I. Physiologic processes
   A. Pancreatic glucagon (M_r = 3485) is a 29-amino-acid polypeptide hormone that is secreted by the α-cells of the pancreas. Glucagon is a member of a superfamily of peptide hormones (collectively called GLP) that influence or regulate several digestive and metabolic processes. Other than glucagon, the major GLP involved in glucose metabolism is GLP-1.[64]
      1. The major role of glucagon is to maintain blood glucose concentrations during fasting. Stimuli for pancreatic glucagon secretion include hypoglycemia, increased amino acids, hypercortisolemia, and probably hypoinsulinemia.
      2. GLP-1 is released from L-cells (large granule cells) of the intestinal mucosa after feeding; it stimulates the release of insulin and reduces postprandial hyperglycemia. Prior to the identification of GLP-1, the hormone was referred to as gut glucagon, which cross-reacted with glucagon in some immunoassays.
   B. Proglucagon is produced by pancreatic α-cells, intestinal L-cells, and the parasympathetic nucleus of the vagus nerve.[64] A variety of factors control the cleavage of proglucagon into glucagon (primarily in the pancreas), GLP-1 (primarily in intestine), and other GLPs.
   C. The major actions of glucagon are illustrated in Fig. 14.1.

II. Analytical concepts
   A. Terms and units
      1. IRG should be reserved for the glucagon that is measured by immunoassays that are

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specific for pancreatic glucagon. Unfortunately, some RIA antibodies also detect other GLPs, especially GLP-1 or gut glucagon. Some authors refer to IRG as glucagon-like immunoreactivity.

2. Unit conversion: pg/mL = ng/L (SI unit, nearest 10 ng/L)[2]

B. Assays
   1. For many years, the gold standard assay for canine IRG was an RIA with Unger's 30K antibody, an antibody that was considered specific for the C-terminal of glucagon.[65] Other investigators have shown the varying degrees of antibody specificity for pancreatic glucagon and glucagon-like immunoreactivity.[66]
   2. One group of investigators described validation studies using an anti-(bovine glucagon) antibody to assess glucagon concentrations in dogs, cats, sheep, cows, and horses. In the fasting state, about 30% of the glucagon-like immunoreactivity was pancreatic glucagon. Their antibody did cross-react with larger molecular forms that had glucagon-like immunoreactivity.[67]
   3. Some investigators use commercial glucagon assays.[68] Results from two commercial glucagon assays differed considerably (> 10-fold in some plasma samples) in a screening evaluation.[69]

C. Samples
   1. Most investigators consider IRG to be very unstable in blood and thus special handling is recommended:[34] Immediately after collection, EDTA-blood is immersed in an ice bath and a protease inhibitor (aprotinin) is added. Plasma is separated from erythrocytes in a 4°C centrifuge and then frozen at –20°C. Samples should be protected from light.
   2. Serum has been used in some studies.[70] There is evidence that the stability of radiolabeled glucagon is not the same in all species.[71] In some assay systems, degradation of radiolabeled glucagon will cause falsely increased measured values.[72]
   3. The presence of arginine can lead to an overestimation of measured [IRG].[73] This finding must be considered when interpreting results of the arginine stimulation tests that have been used to validate IRG assays; i.e., an increased [IRG] after arginine stimulation was used as evidence that the assay was measuring glucagon released from the α-cells.

III. Hyperglucagonemia associated with glucagonomas

A. Because glucagon is a hormone involved in carbohydrate and lipid metabolism, there are many reports of [IRG] in various physiologic and pathologic states. However, [IRG] has very limited use in veterinary diagnostic efforts.

B. Some dogs with pancreatic glucagonomas have a characteristic superficial necrolytic dermatitis, but not all dogs with the dermatologic disorder have glucagonomas.
   1. Increased plasma [IRG] has been reported in some dogs with the disorder.[23,68,74,75]
   2. In a study involving 22 dogs with superficial necrolytic dermatitis, pancreatic neoplasms were not found (19 of 22 had histologic pancreatic examinations) and [IRG] was WRI in 5 evaluated dogs.[76]

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Chapter 15

EXOCRINE PANCREAS AND INTESTINE

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absorptive 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. Examples of intestinal diseases that cause malabsorption include the following:
   1. Inflammatory: histoplasmosis, lymphocytic enteritis, eosinophilic enteritis, pythiosis, giardiasis, protothecosis
   2. Neoplastic: lymphoma
   3. Lymphangiectasia

TRYPSIN-LIKE IMMUNOREACTIVITY (TLI) CONCENTRATION IN DOGS AND CATS

I. Physiologic processes (Fig. 15.1)

II. Analytical concepts
   A. Terms and units
      1. Immunologic assays detect trypsinogen, trypsin, and trypsin bound to protease inhibitors; thus the name TLI. Nearly all TLI is due to trypsinogen.
      2. Units: µg/L (SI units not found)
   B. Assays
      1. Canine and feline [TLI] are measured by species-specific immunoassays. Measuring trypsin's enzymatic activity is not done because of the presence of trypsin inhibitors in serum.
      2. In cats, reference intervals for [TLI] measured by a radioimmunoassay were lower than those measured by an enzyme-linked immunosorbent assay.[2]
   C. Sample: Serum is the preferred sample, but EDTA-plasma or heparinized plasma can be used. Samples should be stored at 4°C or -20°C.[3]

III. Increased trypsin-like immunoreactivity (TLI) concentration (Table 15.2)
   A. Increased release from pancreatic acinar cells
      1. Acinar cell damage due to pancreatitis[4]
         a. Trypsinogen or trypsin released from damaged acinar cells enters blood, probably via lymph and peritoneal fluid.[5]
         b. In an experimental canine pancreatitis study, serum [TLI] tended to parallel serum AMS and LPS activities; i.e., all started to increase within 1 day, remained increased through day 5, and returned to near baseline values at 2 weeks. However, the peak in [TLI] occurred 1–2 days prior to the peak activities of AMS and LPS.[6]
         c. In a prospective study involving 28 cats with clinical signs compatible with pancreatitis, there were not significant differences in serum [TLI] found in cats with no pancreatic lesions (10), cats with pancreatitis and fibrosis (9), cats with pancre-

Table 15.2. Diseases and conditions that cause increased [TLI] in dogs and cats

Increased release of trypsinogen or trypsin from pancreatic acinar cells
[a]Acinar cell damage due to pancreatitis
Stimulated by food intake or cholecystokinin and secretin administration
[a]Decreased GFR (see prerenal, renal, and postrenal azotemia in Chap. 8)

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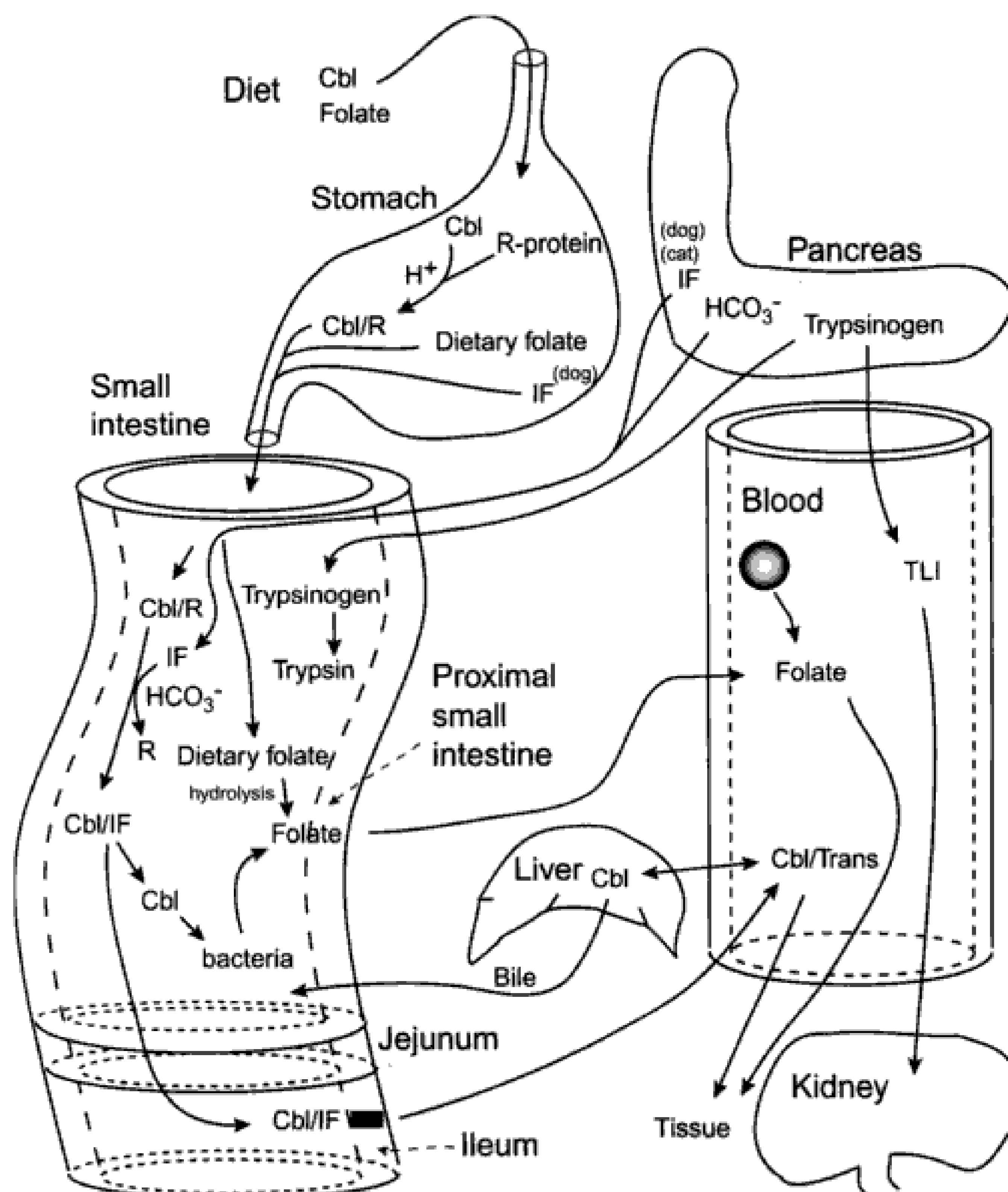

Fig. 15.1. Physiologic processes that influence plasma or serum concentrations of TLI, folate, or cobalamin.

- *TLI:* Most trypsinogen is secreted in enzyme-rich pancreatic secretions into the intestine, where it is converted to trypsin, a potent digestive protease. In health, a small amount of trypsinogen escapes the pancreas and enters blood, in which it can be measured as TLI. Trypsinogen is degraded in kidneys.
- *Cobalamin:* Cobalamin (Cbl) enters the stomach via ingested foods. In the acidic environment, it binds with R-protein that is produced by the gastric mucosa. Cobalamin enters the intestine bound to R-protein, but when it enters the alkaline environment, it detaches from R-protein and binds to intrinsic factor (IF) that is secreted by pancreatic cells (dogs and cats) and gastric mucosa (dogs). 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 and enters enterocytes. When cobalamin enters portal blood, it binds to transcobalamin 2 (Trans), a transport protein. From blood, cobalamin may be used in tissues, stored in liver, or excreted in bile.
- *Folate:* Folate is present in food (e.g., green leafy plants) in a polyglutamate form. After digestion releases it from food, the polyglutamate folate is hydrolyzed to monoglutamate folate at the brush border of the proximal small intestine. After cellular uptake, it is converted to 5-methyltetrahydrafolate, which enters blood and is transported to tissues for biochemical reactions. Enteric bacteria can also produce folate. Folate from erythrocytes lysed during sample collection can increase measured serum folate concentrations.

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reagent contains contaminant R-proteins, they can bind to inactive cobalamin metabolites and give falsely increased values.[13]

C. Sample[3]
   1. Serum is the preferred sample; heparinized or hemolyzed plasma should be avoided.
   2. Samples are stable at 8°C overnight and up to 8 weeks at -20°C.
   3. Falsely low concentrations may occur when cobalamin is degraded by excess exposure to light.

III. Increased serum cobalamin concentration
   A. Increased serum cobalamin concentrations are uncommon. However, disorders or conditions that could increase cobalamin concentrations should be considered when interpreting concentrations that are decreased or WRI.
   B. Diseases or conditions that cause increased serum cobalamin concentrations
      1. Cobalamin supplementation: parenteral or oral
      2. Release from damaged hepatocytes: Because hepatocytes are a storage site for cobalamin, hepatic necrosis could increase serum cobalamin concentrations. The magnitude or duration of the increase is not known.

IV. Decreased serum cobalamin concentration (Table 15.4)
   A. Serum cobalamin concentrations will not be decreased until there is a depletion of body reserves. Depletion occurs because of decreased ileal absorption of cobalamin, but the primary defect may be preabsorptive (i.e., before cobalamin absorption by the ileum).
   B. Diseases or conditions that reduce cobalamin absorption
      1. Preabsorptive defect
         a. EPI[14]
            (1) Failure to secrete HCO_3^--rich fluid into the duodenum results in cobalamin not being released from R-proteins (a pH-dependent event).
            (2) There may be decreased production of intrinsic factor (especially in cats because they apparently lack gastric intrinsic factor). Thus, there is less cobalamin/intrinsic factor complex formation for absorption.
         b. Intestinal bacterial overgrowth (> 10^5 colony-forming units/mL of duodenal juice),[15] especially involving obligate anaerobes, can lower serum cobalamin concentrations by increasing the amount of cobalamin bound to intestinal

Table 15.4. Diseases and conditions that cause decreased serum cobalamin (vit. B_{12}) concentration in dogs and cats

Preabsorptive defect
EPI: pancreatic atrophy, chronic pancreatitis
Intestinal bacterial overgrowth: EPI, impaired gastric acid secretion, enteric disorders
Defective absorption of cobalamin in ileum
Ileal disease: inflammation, resection, villous atrophy (viral, hypersensitivity, cytotoxic drugs)
Inherited absorptive defect in giant schnauzers and border collies
Severe cobalamin deficiency in a cat (probable malabsorption)

Note: Lists of specific disorders or conditions are not complete but are provided to give examples. Falsely low concentrations may occur when cobalamin is degraded by excess exposure to light.

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and nontreated dogs that were fed a folate-deficient diet.[24] The effects of phenytoin on folate concentrations are not known for dogs fed a folate-adequate diet.

2. Sulfasalazine also is reported to lower folate concentrations in people, but similar findings have not been reported for dogs or cats.[25]
3. In theory, sterilization of the intestine with antibiotics may lower serum folate concentrations 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.

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. After bypassing the liver, it passes through the glomerular filtration barrier and is excreted in urine.
  B. In people, xylose does enter the glucuronic acid-xylulose pathway in hepatocytes. It is usually assumed that very little of the absorbed xylose is degraded by hepatocytes of domestic mammals.

II. Analytical concepts
  A. Unit conversion: mg/dL × 0.06661 = mmol/L (SI unit, nearest 0.1 mmol/L)[12]
  B. Assays
    1. There are multiple xylose assays available but clinical laboratories do not commonly offer them.
    2. A colorimetric assay using phyloroglucinol has been used for equine and feline sera.[26] There also are assays that involve deproteination of whole blood, gas chromatography, or enzymatic methods.[27] An o-toluidine assay can measure xylose concentration based on differences in absorbances of the reaction products produced by hexoses (e.g., glucose) and pentoses (e.g., xylose).[28]
    3. The ability of the intestine to absorb sugars other than just xylose (e.g., methyl glucose, rhamnose, and lactulose) can be accomplished by quantitation using high-performance liquid chromatography.[29,30]
  C. Sample
    1. Preferred sample varies with the assay system; generally serum is preferred.
    2. Information on xylose stability was not found.

III. Xylose absorption tests
  A. Basics of procedure
    1. The D-xylose absorption test consists of administering D-xylose orally and then collecting multiple timed blood samples to assess the intestinal ability to absorb D-xylose. Most of the absorbed xylose passes through the liver and enters systemic blood. The peak blood concentration of xylose should reflect the amount of xylose absorbed by the intestine.
    2. Xylose is cleared from plasma by passing through the glomerular filtration barrier; the amount of xylose in urine is related to the amount of xylose absorbed in the intestine.
  B. In dogs and cats
    1. Xylose absorption tests have been used to evaluate small intestinal function of dogs

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Table 15.7. Diseases and conditions that alter xylose absorption curves

Flat xylose absorption curve (decreased absorption)
Reduced absorption due to small intestinal mucosal disease
Incomplete delivery of xylose to intestine: vomiting, delayed gastric emptying
Rapid transit time: diarrhea
Persistent xylose concentration (decreased elimination)
Decreased GFR

and cats.[31-35] In some animals with small intestinal disease, xylose was poorly absorbed and thus there were flat absorption curves.

2. In most of the current textbooks, small animal internists no longer recommend xylose absorption tests, perhaps because of the limited availability of xylose assays or because other methods of characterizing intestinal disease are more fruitful.

C. In horses: Xylose absorption tests have been used to evaluate small intestinal function in horses.[36-40]

IV. Diseases and conditions that alter xylose absorption curves (Table 15.7)

Table 15.8. Other laboratory methods used to evaluate for exocrine pancreatic insufficiency

Laboratory method	Abnormal result	Suggested pathologic state
Azocasein digestion test	Feces failed to digest azocasein	Decreased fecal proteolytic activity
Fecal fat excretion	Increased fecal fat excreted in 24-hr sample	Maldigestion of ingested lipids
Fecal fat, microscopic exam	↑ sudanophilic droplets in feces	Maldigestion of ingested lipids
Fecal starch, microscopic exam	↑ starch granules stained by Lugol's iodine	Maldigestion of ingested starch
Gelatin tube digestion	Feces failed to liquify gelatin adequately	Decreased fecal protease activity
Muscle fibers, microscopic exam	Undigested muscle fibers present	Maldigestion of dietary muscle (protein)
BT-PABA[a] digestion	Decreased plasma PABA concentration or decreased urinary PABA excretion	Decreased chymotrypsin released from pancreas
Plasma turbidity test	Absence of plasma turbidity after ingesting lipid meal but turbidity present after ingesting predigested lipids	Decreased pancreatic lipase activity in intestine
Radiographic film digestion	Feces failed to clear gelatin from film	Decreased fecal proteolytic activity
Starch digestion test	Flat glucose absorption curve	Maldigestion of starch or malabsorption of glucose

Note: Increases in serum amylase and lipase activities can be indicators of active or recent pancreatic acinar cell damage. Serum amylase and lipase activities should not be used to detect EPI because there are extrapancreatic sources of amylase and lipase that can keep serum activity WRI in EPI cases. Serum amylase and lipase activity can be decreased when the animal does not have EPI.

[a] BT-PABA: N-benzoyl-L-tyrosyl-para-aminobenzoic acid (bentiromide).

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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 15.8 and 15.9). 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 15.9. Other laboratory methods used to evaluate intestinal diseases

Laboratory method	Abnormal result	Suggested pathologic state
Bacterial culture (isolation)	Specific isolate (e.g., *Salmonella* sp.)	Bacterial enteritis
Bacterial culture (quantitative)	> 10^5 colony-forming units/ mL of duodenal juice	Small intestinal bacterial overgrowth
Biopsy procedures (incision or excision)	Abnormal cell populations or abnormal tissue structure	Varies with the histologic lesion; typically inflammation or neoplasia
Direct fecal exam	Motile parasites	Giardiasis or amebiasis
Fecal α-1 protease inhibitor assay[a]	Increased fecal α-1 protease inhibitor activity	Protein-losing enteropathy
Fecal flotation test	Parasitic ova, oocytes, or larvae	Parasitism
Fecal occult blood	Positive reaction for heme	Blood in alimentary tract
Fecal sedimentation test	Fluke ova	Parasitism
Hydrogen breath test	Increased hydrogen production	Bacterial overgrowth or carbohydrate malabsorption
Oral glucose tolerance test	Flat absorption curve	Malabsorption of hexoses
Plasma turbidity test with predigested fat	Absence of plasma turbidity	Malabsorption of digested lipids
Stained feces, microscopic exam	Acid-fast organisms	*Cryptosporidia* oocysts or *Mycobacterium* bacilli
	Giardia trophozoites	Giardiasis
	Histoplasma organisms	Histoplasmosis
	Increased leukocytes	Enteritis
	Neoplastic cells	Neoplasia
	Uniform bacilli	Bacterial overgrowth
Starch digestion test	Flat glucose absorption curve	Malabsorption of glucose
Unconjugated bile acids[a]	Increased serum concentrations	Increased deconjugation of bile acids by an overgrowth of intestinal bacteria
Urine indican test	Positive for indican	Increased degradation of tryptophan by enteric bacteria
Urine nitrosonaphthol test	Positive nitrosonaphthol test	Increased bacterial degradation of tyrosine by enteric bacteria

Note: Not a complete list; other special assays are used for specific organisms or pathologic states.

[a] Relatively new procedure; clinical value not established. (Williams DA. 1999. Defining small intestinal disease. In: Proc 17th ACVIM Forum, 466-468. Chicago: American College of Veterinary Internal Medicine.)

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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.

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Chapter 16

LIPIDS

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Table 16.2. Lipoprotein classification, content, and properties of human lipoproteins

	Lipoproteins				
Property	Chylomicron	VLDL	IDL	LDL	HDL
Density (g/mL)	< 0.95	0.95–1.006	1.006–1.019	1.019–1.063	1.063–1.210
Major lipid	Dietary TG	Hepatic TG	Hepatic TG and cholesterol ester	Phospholipid and cholesterol ester	Phospholipid and cholesterol ester
Electrophoretic migration	Origin	Pre-β	β to pre-β	β	α
Diameter (nm)	> 70	25–70	22–24	19–23	4–10
Contribute to serum lactescence	Yes, floats with time	Yes	Maybe	No	No
Major site of formation	Small intestine enterocyte	Hepatocyte	Plasma	Plasma	Hepatocyte
Major sites of degradation or transformation	Plasma and hepatocytes	Plasma	Plasma	Nonhepatic cells, hepatocytes, macrophages	Hepatocyte

Source: Modified from Rifai N, Bachorik PS, Albers JJ. 1999. Lipids, lipoproteins, and apolipoproteins. In: Burtis CA, Ashwood ER, eds. *Tietz Textbook of Clinical Chemistry*, 3rd ed., 809-861. Philadelphia: W.B. Saunders Company.

II. Lipoprotein metabolism (Fig. 16.1)

A. In the context of serum cholesterol and TG concentrations, disorders involving lipoprotein metabolism involve excess synthesis, defective lipolysis, or defective clearance or cellular uptake of lipoproteins.

1. Nascent (newly made) lipoproteins are produced in small intestinal enterocytes (chylomicrons) and in hepatocytes (VLDL and HDL).
2. Lipolysis of lipoproteins (chylomicrons, VLDL, and IDL) occurs on the luminal surface of capillary endothelial cells and is catalyzed by LPL. LPL production and activity is dependent on insulin; insulin allows LPL to "float" from the inner surface of the cell membrane to the outer surface, where it can contact plasma lipoproteins.[2,4] 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.[5,6]
3. Lipoprotein remnants are cleared from plasma by hepatocytes. LDL molecules are removed from plasma by many types of cells. LPL increases LDL uptake by promoting LDL binding to cell receptors.[7]

B. There are many other aspects of lipoprotein metabolism (e.g., mobilization and oxidation of lipids, apoprotein 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.[1,2,5] 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 will be limited.

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Table 16.4. Diseases and conditions that cause hypocholesterolemia

Decreased cholesterol production
Portosystemic shunts in dogs and cats
Protein-losing enteropathy (especially lymphangiectasia)
Other, unknown, or multiple mechanisms
Hypoadrenocorticism

1. Portosystemic shunts: It's reported that about 60%-70% of dogs and cats with portosystemic shunts have hypocholesterolemia.[11] The pathogenesis of the hypocholesterolemia may include decreased cholesterol production due to decreased functional hepatic mass or the inhibition of cholesterol synthesis by bile acids.
2. Protein-losing enteropathy (especially due to intestinal lymphangiectasia):[12-14] The pathogenesis of the hypocholesterolemia may include decreased lipoprotein production because of the catabolic state or, in the case of lymphangiectasia, loss of lipids that are normally produced by enterocytes.
3. Hypoadrenocorticism
   a. In one study involving 17 dogs with hypoadrenocorticism, 13 dogs had hypocholesterolemia.[15] In another study involving 201 dogs, 14 were hypocholesterolemic but 29 had hypercholesterolemia.
   b. As explained in a later section on hyperadrenocorticism, cortisol does influence lipoprotein metabolism, but how hypocortisolemia causes hypocholesterolemia is not established.

TRIGLYCERIDE (TG) CONCENTRATION OF SERUM

I. Physiologic processes
   A. 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.
   B. TG synthesis begins with fatty acids (short, medium, or long chain) and glycerol from carbohydrate metabolism. TG synthesis for lipoproteins occurs in hepatocytes and enterocytes.
   C. When lipoproteins containing apoprotein C-II bind to LPL on endothelial cells, fatty acids are cleaved from TG molecules and enter cells (e.g., adipocytes, myocytes). With repeated processing, the lipoproteins become TG-depleted and thus serum [TG] decreases.

II. Analytical concepts
   A. Terms and units
      1. Serum [TG] represents the total [TG]; the reacting TG molecules were mostly within TG-rich lipoproteins (i.e., chylomicrons, VLDL, IDL). TG molecules are a highly variable population of molecules because of the varied fatty acids that are attached to the glycerol backbone.
      2. Units: mg/dL × 0.01129 = mmol/L (SI unit, nearest 0.05 mmol/L)[8]
   B. Sample
      1. Serum is the preferred sample. Total [TG] is stable at 4°C for 5-7 days, at -20°C for 3 months, and at -70°C for years. Repeated thawing and refreezing should be avoided.[9]

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2. Vacuum tubes with silicone-coated stoppers have been recommended for TG analysis because glycerin from glycerin-coated stoppers 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].

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.
2. High concentrations of free glycerol in serum would produce falsely elevated [TG], but such conditions are rarely recognized.
3. TG concentration in a grossly lipemic sample may exceed an assay's analytical range and thus the sample may need to be diluted prior to analysis.

III. Hypertriglyceridemia

A. Increased serum [TG] indicates there are increased concentrations of lipoproteins that contain TG.

B. Disorders or conditions (Table 16.5)

C. The pathologic processes that result in hypertriglyceridemia are described in a later section on lipoprotein disorders.

D. Increased concentrations of chylomicrons, VLDL, and possibly IDL may cause lactescence or turbid serum and may interfere with spectrophotometric assays. Thus, laboratory personnel may clear the serum (by centrifugation or addition of clearing agents) prior to chemical analysis. When the serum is cleared of the TG-rich lipoproteins, the [TG] in the sample will decrease.

IV. Hypotriglyceridemia: Hypotriglyceridemia is not associated with clinically significant pathologic states.

Table 16.5. Diseases and conditions that cause hypertriglyceridemia

- Increased triglyceride production
  - By hepatocytes
    - [a]Equine hyperlipemia or hyperlipidemia
  - By enterocytes
    - [a]Postprandial hyperlipidemia
- Decreased lipolysis or intravascular processing of lipoproteins
  - Hypothyroidism
  - Nephrotic syndrome
  - Lipoprotein lipase deficiency (rare in cats, very rare in dogs)
- Other, unknown, or multiple mechanisms
  - [a]Acute pancreatitis
  - [a]Diabetes mellitus (see different types in Chap. 14.)
  - High lipid diet
  - Hyperadrenocorticism or excess glucocorticoids
  - Hyperlipidemia in a Brittany spaniel
  - Idiopathic hyperlipidemia of miniature schnauzers

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HYPERLIPEMIA, HYPERLIPIDEMIA , AND HYPERLIPOPROTEINEMIA DISORDERS

I. Terms
  A. *Hyperlipemia, hyperlipidemia,* and *hyperlipoproteinemia* are nearly synonyms.
    1. *Hyperlipemia* and *hyperlipidemia* refer to an increased concentration of lipids in blood, whether it is from hyperlipoproteinemia, hypercholesterolemia, hypertriglyceridemia, or even increased concentrations of free fatty acids.
    2. *Hyperlipoproteinemia* is an increased concentration of lipoproteins in blood. It is the preferred term when measured concentrations of lipoproteins (e.g., LDL, HDL) are 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. The lactescence is primarily due to increased concentrations of the large lipoprotein molecules, i.e., chylomicron and VLDL molecules.

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 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
  C. Secondary hyperlipoproteinemia disorders are acquired disorders involving damaged cells or abnormal hormonal activity. They are relatively common in domestic mammals (see Tables 16.3 and 16.5).

III. Physiologic (postprandial) hyperlipidemia
  A. The hyperlipidemia occurs in monogastric mammals and is due to a hyperchylomicronemia that occurs after intestinal absorption of monoglycerides and fatty acids that have formed from digestion of ingested triglycerides. Hypertriglyceridemia will be present; hypercholesterolemia may not be present.
  B. In dogs and cats, the hyperchylomicronemia peaks by 2-6 hr after a meal and the chylomicrons are usually cleared by 8-16 hr.[5] Delayed clearing of chylomicrons indicates defective lipoprotein metabolism, either a primary or secondary hyperlipidemia.

IV. Primary and possible primary hyperlipidemia disorders in domestic mammals
  A. Idiopathic hyperlipidemia of miniature schnauzers[2,3]
    1. Pathogenesis of the hyperlipoproteinemia is not established; it may be due to a deficiency in LPL activity or an apolipoprotein C-II deficiency.
    2. There is hypertriglyceridemia with or without hypercholesterolemia; there may be concurrent glucose intolerance due to insulin resistance.
  B. Hyperlipidemia in a Brittany spaniel[16]
    1. A 9-year-old male Brittany spaniel was suspected to have "hyperlipemia due to a pro-

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tive energy balance. Hormones or other substances can contribute to the defective metabolic pathways.[42]

a. Catecholamines and glucagon stimulate hormone-sensitive lipase in adipocytes to increase free fatty acid release. Insulin typically inhibits this enzyme and thus decreased insulin activity may remove a negative effector and promote free fatty acid release.
b. Glucocorticoid hormones also stimulate hormone-sensitive lipase.
c. Insulin stimulates LPL for hydrolysis of TG in most lipoproteins, especially the TG-rich chylomicrons and VLDL. Decreased insulin activity reduces the activity of LPL and thus there is reduced intravascular processing of lipoproteins.
d. Progesterone (when increased in pregnancy or lactation) stimulates growth hormone, which creates insulin resistance by causing insulin receptor and postreceptor defects.[43]
e. Hypoglycemia, when present, stimulates glucagon release. Glucagon stimulates hormone-sensitive lipase in adipocytes and thus promotes the liberation of free fatty acids.
f. Either directly or through cytokine release, endotoxins stimulate TG and VLDL synthesis by hepatocytes, and they also can inhibit lipoprotein catabolism.[25,44]
g. Rats in renal failure (created by partial nephrectomy) had defective lipolysis of VLDL molecules and thus hypertriglyceridemia. The agent or agents that produce the defective lipolysis were not identified.[45] It is not known if a similar defect occurs in horses with renal failure.

5. Congenital hyperlipemia in a Shetland pony foal[46]
   a. A Shetland pony neonate had visually lipemic sera, increased total lipid concentration, and hypercholesterolemia. By definition, the foal had a congenital hyperlipidemia, but it may not have been hereditary.
   b. The mare was hyperlipidemic (perhaps associated with pregnancy), so the foal's lipemia may have resulted from the placental transfer of free fatty acids from the mare. The influx of free fatty acids may have stimulated VLDL synthesis sufficiently to cause hyperlipemia in the foal (see adult mechanisms above).

H. High dietary fat intake in dogs
   1. Excessive dietary lipid would result in storage of lipids and potentially obesity. Such dogs may have prolonged postprandial hyperlipidemia because of insulin resistance or other factors.
   2. Extremely elevated fat intake and concurrent inadequate protein intake can lead to defective lipoprotein metabolism and hepatic lipidosis.

OTHER ASSESSMENTS OF LIPIDS

I. TG and cholesterol concentrations in pleural or peritoneal fluid
   A. When an effusion contains chylomicrons, it is called a chylous effusion. If in the pleural space, it is called a chylothorax. Chylous effusions may occur in the peritoneal cavity (chylous ascites). Because chylomicrons are formed only in the intestinal mucosa, the presence of chylomicrons in an effusion indicates the leakage of chylomicron-containing lymph (chyle) from lymphatic vessels.
      1. Historically, chylothorax was considered to result from a damaged thoracic duct. However, many diseases that result in an increased systemic venous pressure are known to cause chylothorax. These diseases include right heart failure, mediastinal

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D. A pleural or peritoneal effusion could have a high [TG] if TG-rich plasma entered the body cavity (e.g., with hemorrhage). If the [TG] of the effusion and the serum are each > 100 mg/dL, then the diagnostician should consider nonchylous effusions.

E. The term *pseudochylous* has been used to describe milky-white to yellow effusions that have high cholesterol concentrations due to the degradation of cell membranes. Human data indicate that if such effusions occur in animals, they should be cholesterol-rich and TG-poor, and therefore distinguishable from chylous effusions.

II. Standing plasma test for chylomicrons (also called a refrigeration test)

A. Because of their high TG content and thus low density, chylomicrons will float to the surface of a serum or plasma sample if the sample is undisturbed.

B. A 16-hr-standing test has been used to detect chylomicrons. In the procedure, 2 mL of plasma is placed in a small glass tube and then examined after 16 hr of refrigeration at 4°C. The presence of a creamy layer on the plasma sample indicates the presence of chylomicrons.

C. However, the 16-hr-standing test has a relatively poor ability to detect 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-hr-standing test.[49] Similar results were found when the standing test was compared to ultracentrifugation.[50] The presence of a creamy layer in the standing test is specific for chylomicrons, but many samples that contain chylomicrons will not have a detectable lipid layer.

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RESPONSE AND SUPPRESSION TESTS

I. Thyroid stimulating hormone (TSH) response test
   A. Poor or inadequate response to TSH stimulation was the "gold standard" for establishing thyroid hypoplasia (hypothyroidism) for many years. Because an approved pharmacologic form of bovine TSH is no longer available, the TSH response test is rarely used for clinical investigations. Basic aspects of the TSH response test are provided to assist in the understanding of published reports on canine hypothyroidism.
   B. A TSH response test assesses the ability of the thyroid gland to produce T_4 after being stimulated by TSH. Several protocols have been recommended; the amount of TSH and sample collection times vary between feline, canine, and equine protocols.
   C. Example of a canine TSH response test
      1. Procedure: Sample for a basal [tT_4] is collected; bovine TSH is administered IV (0.1 units/kg body weight with a maximum of 5 units); a blood sample is collected 6 hr after TSH administration.
      2. Interpretation guidelines[21]
         a. Euthyroid dogs
            (1) Basal [T_4] in healthy dogs is expected to be 1.0–4.0 µg/dL. In nonthyroidal illness, it may be < 1.0 µg/dL.
            (2) Post-TSH [tT_4] > 3.0 µg/dL
         b. Borderline results: post-TSH [tT_4] = 1.5–3.0 µg/dL
         c. Hypothyroid dogs: basal and post-TSH [tT_4] < 1.5 µg/dL

II. Thyrotropin releasing hormone (TRH) response test
   A. The TRH response test indirectly assesses thyroid function by causing the release of TSH that stimulates release of T_4.
   B. In dogs, the TRH response test is not as good as the TSH response test in detecting hypothyroidism.[37,38] Some euthyroid dogs (as classified by the TSH response test and clinical signs) appear to be hypothyroid dogs by the TRH response test (positive predictive value of 50%).[39] The TRH response test does not reliably differentiate primary from secondary hypothyroidism because TRH-induced TSH release is diminished in secondary hypothyroidism and in most primary hypothyroid dogs.[21]
   C. TRH is not approved for use in the horse.
   D. The TRH response test is not needed to diagnose hyperthyroidism in cats, but it has been used.[40]
      1. The TSH and serum T_4 responses to TRH are expected to be blunted or absent in hyperthyroid cats because of feedback inhibition on the pituitary gland. Serum [tT_4] in a healthy cat is expected to double in the post-TRH sample.
      2. Advantages of the TRH response test over the T_3-suppression test are that it is shorter (4 hr versus 3 days) and does not require owners to pill their cat. Disadvantages of the TRH response test are the side effects of TRH administration (salivation, vomiting, tachypnea, and defecation).

III. Triiodothyronine (T_3) suppression test
   A. Cats
      1. Most cats with clinical hyperthyroidism have hyperthyroxemia and thus additional diagnostic testing is not needed. However, some cats with hyperthyroidism have a basal [tT_4] that is WRI or only slightly increased, perhaps because of variable secre-

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tion of T_4 from hyperfunctional thyroid glands, a nonthyroidal disease that is lowering [tT_4], or other reasons. For these animals, a T_3 suppression test should differentiate the hyperthyroid cats (those with defective hypothalamus-pituitary-thyroid regulation) from euthyroid cats.[41,42]

2. Basics of the procedure
   a. Collect serum for basal [tT_4] and basal [tT_3]; freeze for submission with later serum samples.
   b. Owners administer T_3 (liothyronine) starting the next morning at 25 µg q8hr for 2 days.
   c. Within 2–4 hr of the final and 7th dosage of T_3, the cat is returned to the clinic and serum is collected for post-T_3 [tT_4] and [tT_3].
   d. The basal and post-T_3 sera are submitted to a laboratory for determination of [tT_4] and [tT_3].
3. Expected results
   a. The post-T_3 [tT_3] should be higher than the basal [tT_3]. If it is not, then failure of suppression may be due to failure to administer T_3.
   b. Interpretation guidelines of post-T_3 [tT_4] (Fig. 17.3)[41]
      (1) Post-T_3 [tT_4] > 20 nmol/L (> 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 (< 1.6 µg/dL) demonstrates a suppression of T_4 secretion and thus indicates that the cat does not have a thyroid adenoma.

B. Horses
   1. 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.

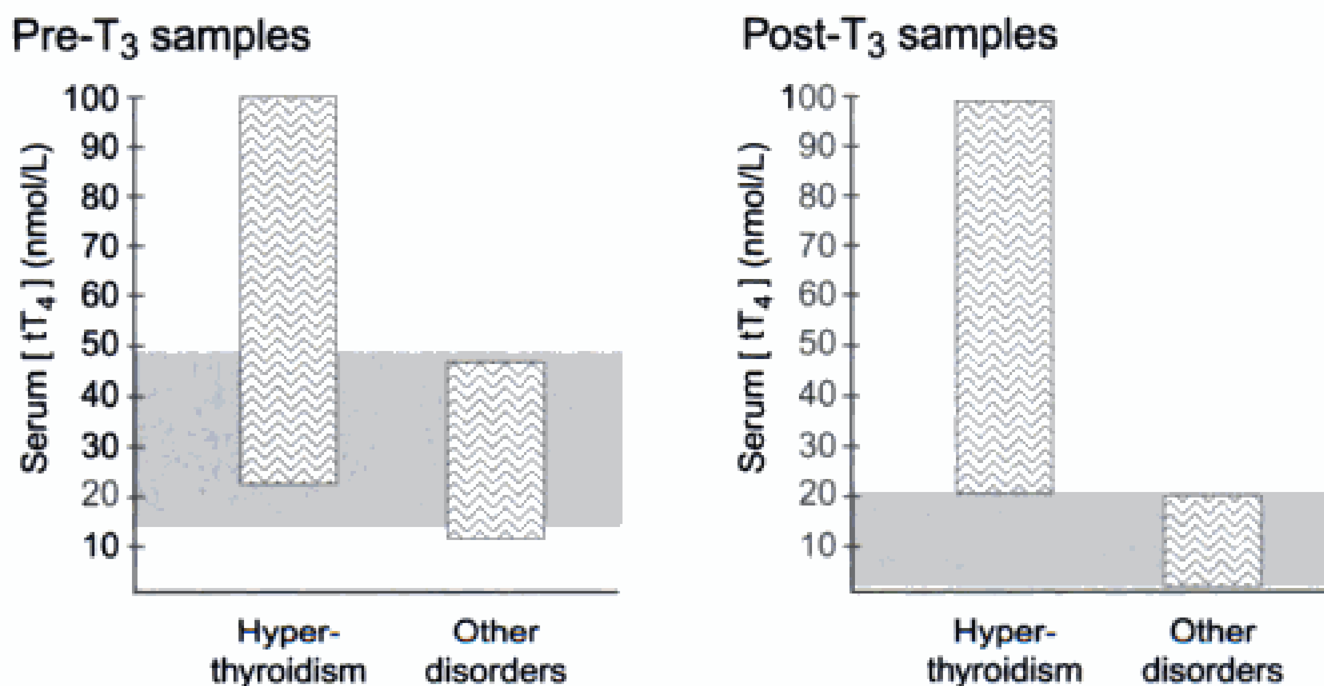

Fig. 17.3. T_3 suppression test results in cats (liothyronine, 25 µg per os, q8hr for 7 doses).
In cats with hyperthyroidism, there was a failure to suppress [T_4] below 20 nmol/L. In cats with other disorders, the [T_4] in post-T_3 samples were < 20 nmol/L. Hyperthyroidism was diagnosed in 77 cats based 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 gray-shaded area represents the [tT_4] found in 44 clinically healthy cats. (Graph constructed from published data.[41])

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2. Basics of the procedure
   a. EDTA-plasma is collected for basal [tT_4] and basal [tT_3]; freeze for submission with later samples.
   b. T_3 (2.5 mg diluted in 5 mL saline) is administered at 8:30 a.m. and 6:00 p.m. for 3 days and at 8:30 a.m. on the 4th day.
   c. EDTA-plasma is collected on days 2 to 4 before the administration of T_3 and on days 6, 7, and 9.
   d. Basal and post-T_3 plasma are submitted to a laboratory for determination of [tT_4] and [tT_3].
3. Expected results[13]
   a. In three clinically healthy horses, plasma [tT_4] values were suppressed (< 4 ng/mL) for at least 5 days after the last dose of T_3.
   b. In a horse with a thyroid adenoma, plasma [tT_4] remained increased (> 24 ng/mL) during and after administration of T_3.
   c. [tT_3] should be increased in samples collected on days 2–4.

THYROXINE:THYROID STIMULATING HORMONE (T_4:TSH) AND FREE THYROXINE:THYROID STIMULATING HORMONE (fT_4:TSH) RATIOS IN DOGS

I. Because of the physiologic relationships between T_4, fT_4, and TSH secretion, T_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). The T_4:TSH ratio may also decrease in secondary hypothyroidism because the [T_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., [tT_4] of 10 nmol/L and a [TSH] of 0.5 ng/mL would result in a ratio of 20 nmol T_4/µg TSH (or mmol/g)
   B. T_4:TSH ratios in one study showed that hypothyroid dogs usually had lower ratios (1–66; 10 of 11 ratios < 30) than healthy euthyroid dogs (36–173) and sick euthyroid dogs (45–2114, a ratio > 4000 was an obvious outlier).[43]
   C. T_4:TSH and fT_4:TSH ratios in 1999 study[44]
      1. Using a T_4:TSH ratio cutoff of 17.3, the T_4:TSH ratio had a diagnostic sensitivity for hypothyroidism of 86.7% and a diagnostic specificity of 92.2%.
      2. Using a fT_4:TSH ratio cutoff of 7.5, the fT_4:TSH ratio had a diagnostic sensitivity for hypothyroidism of 80.0% and a diagnostic specificity of 97.4%.
   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 (due to detection limit) 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 17.4) [32-34,43-50]
   B. [tT_4] can be used to help monitor thyroxine therapy. For dogs receiving appropriate thyroxine supplementation, [tT_4] is expected to be high-normal to increased 4–6 hr after thyroxine administration. TSH concentrations should be WRI.[21]

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Table 17.4. Interpretation of thyroid profile results in dogs

$[tT_4]$	$[fT_4]_{ed}$	[TSH]	TgAA titer	Interpretation
WRI	—	—	—	Rules out hypothyroidism unless tT_4 is falsely increased by T_4AA
↓	↓	↑	positive	Primary hypothyroidism due to lymphocytic thyroiditis
↓	↓	↑	negative	Primary hypothyroidism due to thyroid atrophy
↓	↓	WRI[a]	negative	Secondary hypothyroidism due to pituitary gland dysfunction Sick euthyroidism, nonthyroidal illness[b] Hypothyroxemia due to effects of drugs[c]
↓	WRI-↑[d]	WRI-↑	negative	Nonthyroidal illness (sick euthyroidism)
WRI-↑	↓	↑	positive	Primary hypothyroidism (lymphocytic thyroiditis) with interfering T_4AA
WRI	WRI	WRI	positive	Thyroiditis without thyroid dysfunction
WRI	WRI	↑	positive	Thyroiditis with compensatory increase in TSH production
WRI	WRI	↑	negative	Potentially responding after withdrawal of suppressive drugs or after a nonthyroidal illness[e]

Note: Thyroxine concentrations near the reference limits (borderline results) should be interpreted cautiously.

[a] The canine TSH assay lacks an adequate analytical range to accurately measure decreased [TSH]; therefore, the lower limit of a TSH reference interval cannot be reliably established.

[b] *Source:* Scott-Moncrieff JCR, Nelson RW, Bruner JM, Williams DA. 1998. Comparison of serum concentrations of thyroid-stimulating hormone in healthy dogs, hypothyroid dogs, and euthyroid dogs with concurrent disease. J Am Vet Med Assoc 212:387-391.

[c] Sulfamethoxazole/trimethoprim, prednisolone, phenobarbital.

[d] $[fT_4]_{ed}$ will vary; depends on where the illnesses or drugs interfere with thyroid hormone production (see text).

[e] *Source:* Mooney CT. 1999. Canine TSH—A help or a hindrance? In: *Proc 17th ACVIM Forum,* 456-457. Chicago: American College of Veterinary Internal Medicine.

C. To reassess a diagnosis of hypothyroidism via hormone assays in a dog receiving thyroxine supplementation, thyroxine should be discontinued for at least 4 weeks to allow thyroid function to return.[1]

II. Major patterns of $[T_4]$ and $[fT_4]$ in cats (Table 17.5)

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Table 17.5. Interpretation of thyroxine concentrations in cats

Clinical history and signs suggest:	$[tT_4]$	$[fT_4]_{ed}$	Interpretation
Hyperthyroidism	↑	—	Strong evidence of hyperthyroidism (a concurrent ↑ $[fT_4]$ provides additional evidence but is typically not needed)
	↓-WRI	WRI-↑	May be hyperthyroidism or nonthyroidal illness; T_3-suppression test recommended
Euthyroidism	WRI	WRI	No evidence of thyroid dysfunction
	↑	—	Could be early hyperthyroidism, normal random variation, or inappropriate reference interval; a confirmatory $[tT_4]$ or a T_3-suppression test is needed before diagnosing hyperthyroidism
	↓	—	Probably nonthyroidal illness altering $[tT_4]$ or $[fT_4]$
	↓-WRI	↑	Probably nonthyroidal illness altering $[tT_4]$ or $[fT_4]$
	↓	WRI	Probably nonthyroidal illness altering $[tT_4]$ or $[fT_4]$
Hypothyroidism	↓	WRI-↑	Nonthyroidal illness causing sick euthyroidism
	↓	↓	Probable primary hypothyroidism; could be secondary hypothyroidism or changes due to nonthyroidal illness or drug therapy

Note: Thyroxine concentrations near the reference limits (borderline results) should be interpreted cautiously.

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Chapter 18

ADRENOCORTICAL FUNCTION

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Table 18.1. Abbreviations and symbols in Chapter 18

»	symbol in tables to indicate relatively common disease or condition
[x]	concentration of x; x = analyte
ACTH	adrenocorticotrophic hormone (corticotropin)
$(Cort:Crt)_u$	urine cortisol to creatinine
CRH	corticotropin-releasing hormone
EDTA	ethylenediaminetetraacetic acid
FAN	functional adrenal neoplasia
HDDST	high-dose dexamethasone suppression test
LDDST	low-dose dexamethasone suppression test
o,p'DDD	1, 1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane
PDH	pituitary-dependent hyperadrenocorticism
PU/PD	polyuria/polydipsia
RIA	radioimmunoassay
SI unit	Système International d'Unités
WRI	within reference interval

PHYSIOLOGIC PROCESSES

I. Regulation of cortisol and aldosterone secretion (Fig. 18.1)
   A. CRH from the hypothalamus stimulates the production and release of pituitary ACTH and other hormones. Low cortisol concentrations promote secretion of CRH and ACTH; high concentrations of cortisol inhibit secretion of CRH and ACTH.
   B. ACTH stimulates the production and release of cortisol, aldosterone, and other steroid compounds from the adrenal glands. The adrenal gland cortices produce most circulating cortisol. Aldosterone secretion is also stimulated by angiotensin II, hyperkalemia, and hyponatremia but is inhibited by atrial natriuretic peptide. The secreted aldosterone stimulates the renal retention of Na^+ and Cl^- and excretion of K^+ and H^+.
   C. Peak secretion of cortisol occurs in the morning in the dog and horse, but in the evening in the cat. The degree of daily variation is minimal in the domesticated mammals,[1] but

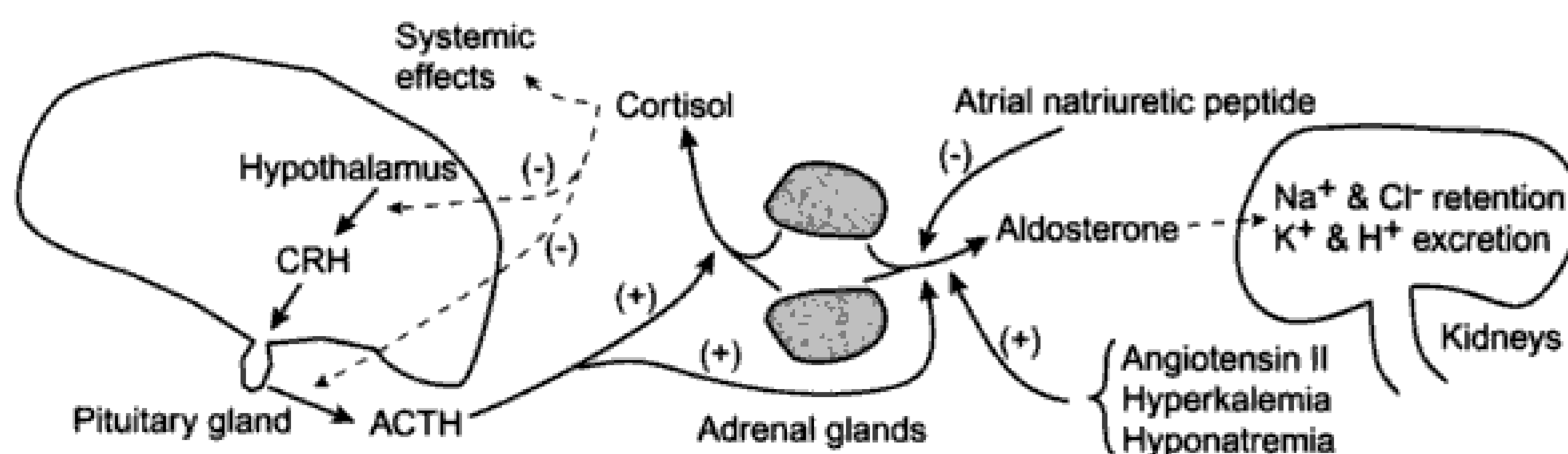

Fig. 18.1. Regulation of cortisol and aldosterone secretion.
- CRH released from the hypothalamus stimulates the production and release of ACTH from the pituitary gland. ACTH stimulates the production and release of cortisol and aldosterone from the adrenal gland cortices. Increasing cortisol concentrations inhibit the secretion of CRH and ACTH.
- Multiple factors promote the aldosterone release 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^+.

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may need to be considered when interpreting diagnostic tests in horses (average about 3.0 μg/dL at midnight; averages 4.5–5.0 μg/dL from 8:00 a.m. to 4:00 p.m.).[2]

D. Nearly all cortisol released from the adrenal glands becomes bound to plasma proteins.[1] In dogs, about 40% of cortisol is bound to transcortin, 50% to albumin, and the remainder (5%–10%) is free. The half-life of cortisol is about 1.5 hr in dogs and less in cats.

E. Cortisol binds to receptor proteins in cells. The cortisol-receptor complex initiates synthesis of hormone and cytokine receptors and other proteins involved in gluconeogenesis, protein catabolism, lipolysis, immune responses, and H_2O balance.

F. Most cortisol is removed from plasma by hepatocytes, but there is also urinary excretion of cortisol and cortisol metabolites.

ANALYTICAL CONCEPTS

I. Unit conversion[3]

A. Cortisol: μg/dL × 10 = ng/mL; μg/dL × 27.6 = nmol/L (SI unit, nearest 10 nmol/L)

B. Aldosterone: ng/dL × 10 = pg/mL; ng/dL × 27.74 = pmol/L (SI unit, nearest 10 pmol/L)

C. ACTH: ng/L = pg/mL; pg/mL × 0.2202 = pmol/L (SI unit, nearest 1 pmol/L)

II. Sample

A. Cortisol

1. Serum or EDTA-plasma may be used.
2. Stability of cortisol concentrations in EDTA-plasma is better than in sera and better in cold samples than in warm ones. Therefore, samples (especially sera) should be shipped with ice packs.[4]
3. It is occasionally written that serum or plasma should be collected from blood quickly because of the uptake of cortisol by erythrocytes. Erythrocytes do bind cortisol that is added to blood,[5] but cortisol should already be distributed to plasma and erythrocytes in patient blood samples. There was no difference between measured cortisol concentrations in plasma samples removed from cells either 10 min or 40 hr after blood collection.[6]

B. Aldosterone

1. Serum or heparinized plasma may be used.
2. Aldosterone concentrations are stable for a week at 2°–8°C and for at least 2 months at –20°C.[7]

C. ACTH

1. EDTA-plasma is preferred (addition of a protease inhibitor such as aprotinin is recommended to reduce degradation). Plasma should be removed from the erythrocytes 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.[8] If not analyzed the day of collection, the sample should be frozen and shipped to the reference laboratory in a dry ice shipment. Equine ACTH concentrations are stable in EDTA-plasma (without aprotinin) for 3 hr at 19°C.[9]

III. Principles and assay procedures

A. Cortisol

1. There are several commercial cortisol assays available; radioimmunoassays are the most common but enzyme immunoassays are also used.

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II. $(\text{Cort:Crt})_u$ ratio should be calculated using molar concentrations of urine cortisol and creatinine (Eq. 18.1). A markedly different ratio would result if cortisol and Crt concentrations were expressed as µg/dL and mg/dL, respectively. Instead of stating the true ratio (e.g., 20×10^{-6}), sometimes it is shortened to "20" in clinical jargon.

$$(\text{Cort:Crt})_u \text{ ratio} = \frac{\text{urine cortisol concentration}}{\text{urine creatinine concentration}} \quad (18.1)$$

with cortisol in nmol/L & creatinine in mmol/L
Example: urine cortisol concentration = 200 nmol/L
urine creatnine concentration = 10 mmol/L

$$(\text{Cort:Crt})_u \text{ ratio} = \frac{200 \text{ nmol/L}}{10 \text{ mmol/L}} = \frac{200 \text{ nmol/L}}{10 \times 10^{6} \text{ nmol/L}} = 20 \times 10^{-6}$$

III. Conclusions from published reports (Fig. 18.2)[18,19]

A. A $(\text{Cort:Crt})_u$ ratio that is WRI is strong evidence that a dog does not have hyperadrenocorticism. However, increased $(\text{Cort:Crt})_u$ ratio can be found in dogs with hyperadrenocorticism and in many dogs with nonadrenal disorders.

B. $(\text{Cort:Crt})_u$ ratio in dogs has high diagnostic sensitivity for hyperadrenocorticism (few false negatives) but has a poor positive predictive value because increased $(\text{Cort:Crt})_u$ ratio is frequently found in dogs without hyperadrenocorticism (many false positives).

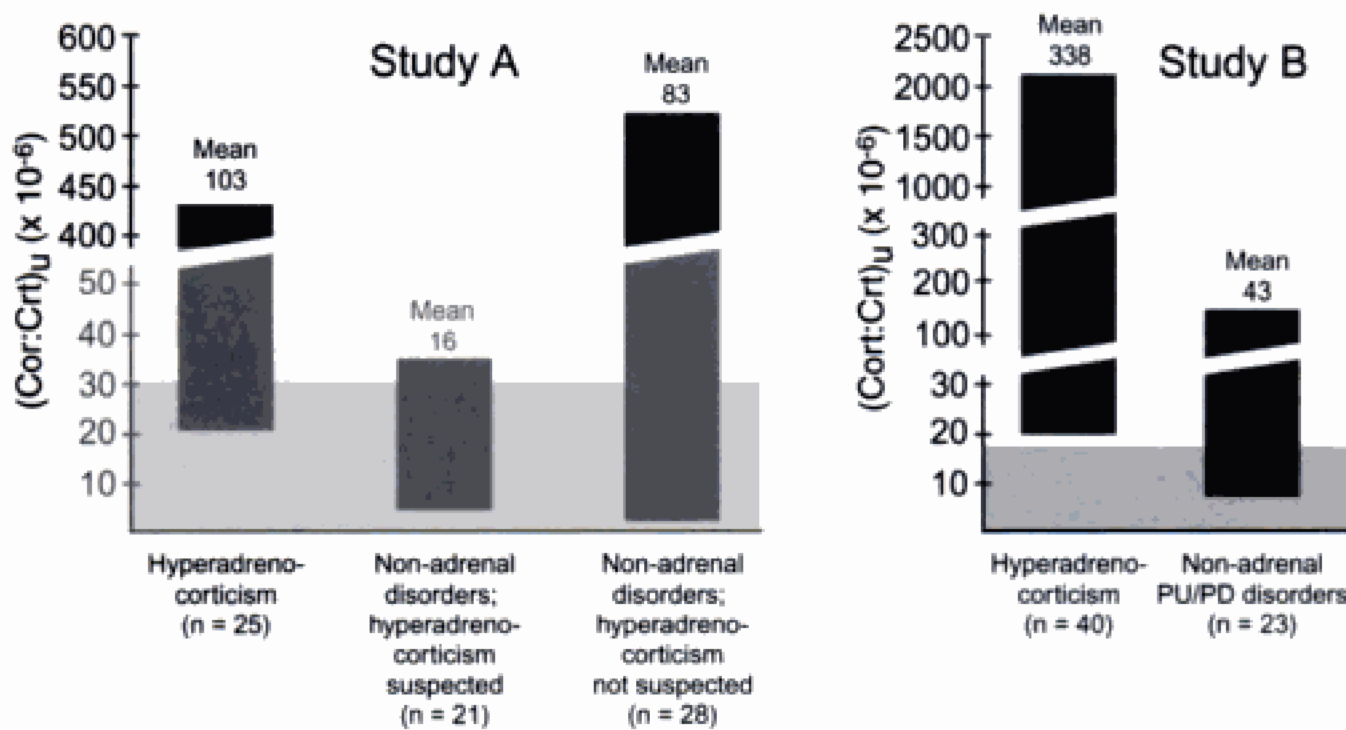

Fig. 18.2. $(\text{Cort:Crt})_u$ ratios in two studies.

- In *Study A*, $(\text{Cort:Crt})_u$ ratios from three groups of dogs were compared to a group of healthy dogs (background gray region; mean = 13×10^{-6}, n = 31). Of 25 dogs with hyperadrenocorticism (21 PDH and 4 FAN), 23 had increased $(\text{Cort:Crt})_u$ ratios. Of 21 dogs with nonadrenal disorders (renal insufficiency, liver disease, pyelonephritis, hypothyroidism, bronchitis, and diabetes insipidus) but in which hyperadrenocorticism was suspected, only 1 had an increased $(\text{Cort:Crt})_u$ ratio. However in 28 dogs with moderate to severe nonadrenal disorders (gastrointestinal, renal, lower urinary tract, liver, neurologic, immune-mediated, cardiac, traumatic, and infectious diseases), 22 had increased $(\text{Cort:Crt})_u$ ratios.[40]
- In *Study B*, $(\text{Cort:Crt})_u$ ratios from dogs with hyperadrenocorticism (36 with PDH and 4 with FAN) and other polyuria/polydipsia disorders (diabetes insipidus, hypercalcemic disorders, liver disease, pyometra, and diabetes mellitus) were compared to $(\text{Cort:Crt})_u$ ratios found in healthy dogs (background gray region, mean = 6×10^{-6}, n = 20). All 40 dogs with hyperadrenocorticism had increased $(\text{Cort:Crt})_u$ ratios, but so did 18 of the 23 polyuria/polydipsia dogs that did not have hyperadrenocorticism.[41]

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d. If a HDDST is done in a dog with nonadrenal illness, cortisol production is expected to be decreased sufficiently to indicate a suppression of ACTH secretion.
3. Expected patterns of LDDST and HDDST results for individual dogs
a. In healthy dogs, cortisol concentrations will suppress to < 1.4 μg/dL (< 40 nmol/L) with both tests (both 4-hr and 8-hr samples).
b. If hyperadrenocorticism is due to PDH, cortisol concentrations are usually suppressed to < 1.4 μg/dL (< 40 nmol/L) with the HDDST, and often with the LDDST also.
c. If hyperadrenocorticism is due to FAN, cortisol concentrations are expected to be ≥ 1.4 μg/dL (≥ 40 nmol/L) in nearly all post-dexamethasone samples.

C. Interpretation of dexamethasone suppression tests
1. Table 18.4 contains examples of cortisol concentrations from dexamethasone suppression tests.
2. Interpretations for Table 18.4 were made using Fig. 18.4 as a guide. Best interpretations are made when results are interpreted in context of historical or physical findings.

D. Adrenocorticotrophic hormone (ACTH) stimulation test
1. Procedure (may begin at any time of day)[24]
a. A sample for pre-ACTH cortisol concentration is collected.

Table 18.4. Example results of dexamethasone suppression tests in dogs

	LDDST Cortisol (μg/dL)[a]			HDDST Cortisol (μg/dL)		
	Pre-dex.[b]	4 hr Post-dex.	8 hr Post-dex.	Pre-dex.	4 hr Post-dex.	8 hr Post-dex.
Healthy dogs	0.5–6.0	< 1.4	< 1.4	0.5–6.0	< 1.4	< 1.4
Dog 1[c]	4.5	3.0	3.5	3.5	3.3	2.9
Dog 2[d]	7.0	1.0	3.8	6.2	0.8	0.6
Dog 3[e]	5.0	0.9	0.6	5.5	0.5	0.3
Dog 4[f]	6.5	3.2	2.8	4.5	0.8	0.4
Dog 5[g]	0.2	0.1	0.1	—	—	—

[a] Conversion to SI units: μg/dL × 27.6 = nmol/L (round to nearest 10).

[b] dex. = dexamethasone.

[c] Dog 1 had either PDH or FAN. In the LDDST, inadequate suppression indicates PDH, FAN, or nonadrenal disorders. In the HDDST, inadequate suppression is more likely in FAN but can be found with PDH; adequate suppression is expected in nonadrenal disorders.

[d] Dog 2 had adrenal hyperplasia due to PDH or a hyper-responsive adrenal gland due to nonadrenal disorder. In the LDDST, 4-hr suppression and then escape at 8 hr suggests PDH or nonadrenal disorders. In the HDDST, suppression can be found with PDH, is expected with nonadrenal disorders but is not consistent with FAN.

[e] Dog 3 was healthy, had PDH, or had a nonadrenal disorder. In the LDDST, suppression can be found with health, PDH, and nonadrenal disorders. In the HDDST, suppression can be found with health, PDH, and nonadrenal disorders; suppression is not expected with FAN.

[f] Dog 4 had adrenal hyperplasia due to PDH or a nonadrenal disorder. In the LDDST, failure to suppress < 1.4 μg/dL (< 40 nmol/L) could be PDH, FAN, or nonadrenal disorder. In the HDDST, suppression can be found with PDH and is expected with nonadrenal disorders; suppression is not consistent with FAN.

[g] Dog 5 had a hypoadrenal state. In the LDDST, the pre-dex. hypocortisolemia indicates a hypoadrenal state that could be primary, secondary, or iatrogenic. The HDDST is not needed in such cases.

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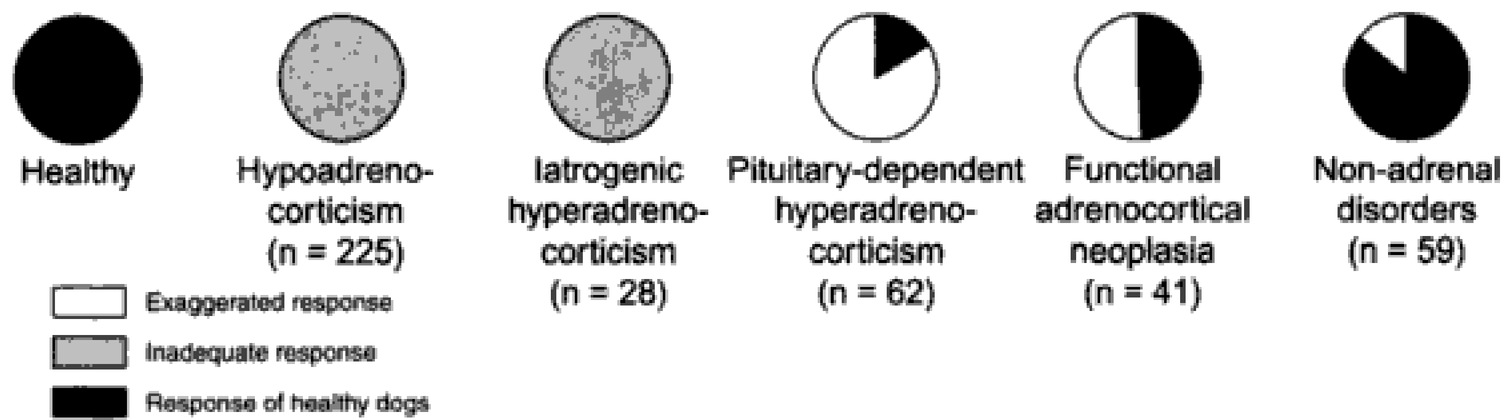

Fig. 18.5. Responses from canine ACTH stimulation tests.

- Criteria used to determine appropriate, inadequate, or exaggerated responses to ACTH stimulation varied between publications. For each set of extracted data, the authors' criteria were used.
- In all hypoadrenocorticism cases, there were inadequate responses to ACTH stimulation. The 225 cases included 220 cases of primary idiopathic hypoadrenocorticism and 5 cases of secondary hypoadrenocorticism.[43]
- In all cases of iatrogenic hyperadrenocorticism, adrenocortical atrophy resulted in inadequate responses to ACTH stimulation.[46]
- In 84% of PDH cases and in 51% of FAN cases, there were exaggerated responses to ACTH stimulation.[45,47-52]
- In 14% of dogs with nonadrenal illnesses, there were exaggerated responses to ACTH stimulation.[45]

b. Synthetic ACTH (Cortrosyn®) is given IM at 0.25 mg/dog, or porcine ACTH (Cortigel-40) is given at 2.2 IU/kg (1 IU/lb) IM.

c. A post-ACTH sample is collected 1 hr later if synthetic ACTH is given (2 hr if porcine ACTH is used).

2. Expected results (Fig. 18.5)
   a. Healthy dogs
      (1) ACTH should stimulate the production and release of cortisol from the adrenal cortices.
      (2) Expected pre- and post-ACTH concentrations of cortisol are different in published reports and reference laboratories. Reference intervals provided with patient results should be used for interpretation guidelines. Usually, post-ACTH concentrations of cortisol are < 17 µg/dL (470 nmol/L).
   b. Hypoadrenocorticism
      (1) Dogs with primary, secondary, or iatrogenic adrenocortical hypoplasia or atrophy are expected to have fewer or no responsive cells and thus post-ACTH cortisol concentrations increase inadequately or not at all.
      (2) Pre- and post-ACTH cortisol concentrations < 1.0 µg/dL (< 30 nmol/L) indicate adrenocortical atrophy, destruction, or hypoplasia.
   c. Hyperadrenocorticism
      (1) From 60% to 80% 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. It is not clear why exaggerated responses do not occur in all dogs with hyperplastic glands. Some of the adrenocortical neoplasms may not respond because the neoplastic cells have defective receptors or other signaling pathways.
      (2) The decision limit that represents an exaggerated response varies between studies and ranges from 18 to 22 µg/dL (500–610 nmol/L).
   d. Nonadrenal illnesses

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(1) Some dogs with disorders other than PDH or FAN may have exaggerated responses to ACTH.
(2) Typically, these animals are thought to have stress-induced hypercortisolemia.
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 causes destruction of the zona fasciculata and the zona reticularis.
(2) ACTH stimulation tests can be used to monitor destruction of adrenocortical tissue. Typically, the goal of o,p'DDD treatment is to get the post-ACTH cortisol concentration to be < 5 μg/dL (< 140 nmol/L) but not cause sufficient destruction to create a hypoadrenocortical state.

II. Cats
A. Low-dose dexamethasone suppression test (LDDST)
1. Procedure: Basically the same procedure as for canine LDDST except dexamethasone is given at either 0.01 or 0.015 mg/kg IV.
2. Expected results[20]
a. In healthy cats, the 4-hr and 8-hr cortisol concentrations are usually < 1.0 μg/dL (< 30 nmol/L), but about 15%–20% fail to suppress. Cats with nonadrenal illness may also fail to suppress. Therefore, the LDDST has poorer diagnostic specificity than the HDDST (for which very few cats fail to suppress) and thus is not recommended. Post-dexamethasone cortisol concentrations of 1.0–1.4 μg/dL (30–40 nmol/L) are considered borderline results.
b. In cats with hyperadrenocorticism, the 4-hr and 8-hr cortisol concentrations are expected to be > 1.5 μg/dL (> 40 nmol/L).
B. High-dose dexamethasone suppression test (HDDST)
1. Procedure
a. The initial protocol is the same as the canine HDDST.
b. Cats may escape the suppressive effects of dexamethasone faster than dogs, so suppression may be seen only if samples are collected at 2 hr, 4 hr, and 6 hr post-dexamethasone.[25]
2. Expected results[20]
a. In healthy cats, the 4-hr and 8-hr cortisol concentrations are < 1.0 μg/dL (< 30 nmol/L); very few fail to suppress. Post-dexamethasone cortisol concentrations of 1.0–1.4 μg/dL (30–40 nmol/L) are considered borderline results.
b. In about 90% of cats with hyperadrenocorticism, the 4-hr and 8-hr cortisol concentrations were > 1.5 μg/dL (> 40 nmol/L).
C. Adrenocorticotrophic hormone (ACTH) stimulation test
1. Procedure (may begin at any time of day)
a. A sample for pre-ACTH cortisol concentration is collected.
b. Synthetic ACTH (Cortrosyn) is given IM at 0.125 mg/cat.
c. Post-ACTH samples are collected at 30 min and 60 min.
2. Expected results[24]
a. Healthy cats should have pre-ACTH cortisol concentrations of 1.0–6.0 μg/dL (30–170 nmol/L) and post-ACTH cortisol concentrations < 13.0 μg/dL (< 360 nmol/L).
b. Cats with hyperadrenocorticism may have pre-ACTH cortisol concentrations WRI or increased, and 30-min or 60-min post-ACTH cortisol concentrations

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> 16 μg/dL (> 440 nmol/L). However, only about 40%–50% of cats with hyperadrenocorticism have an exaggerated response.[20]

III. Horses
   A. Dexamethasone suppression test[26]
      1. Procedure, overnight method:[2]
         a. A sample for pre-dexamethasone cortisol concentration is collected between 4:00 p.m. and 6:00 p.m.
         b. Dexamethasone is given at 40 μg/kg IM.
         c. Post-dexamethasone samples are collected at noon the next day (about 19 hr post-dexamethasone).
      2. Expected results
         a. Healthy horses
            (1) Guideline for adequate suppression: post-dexamethasone cortisol concentration < 1.0 μg/dL (< 30 nmol/L)
            (2) In 34 horses, cortisol concentrations were decreased 8–24 hr post-dexamethasone; all had concentrations < 1.0 μg/dL at 20–24 hr post-dexamethasone.
         b. Hyperadrenocorticism
            (1) Guideline for inadequate suppression: post-dexamethasone cortisol concentration > 1.0 μg/dL (> 30 nmol/L)
            (2) In 52 horses with PDH, the maximal suppression was seen 8–12 hr post-dexamethasone; all had cortisol concentrations > 1.0 μg/dL at 20–24 hr post-dexamethasone.
   B. Adrenocorticotrophic hormone (ACTH) stimulation test
      1. Multiple ACTH compounds have been used for stimulation tests but nearly all studies included a pre-sample and a 2-hr post-ACTH sample.
      2. Results expected for healthy and PDH horses if ACTH gel is given (1 U/kg IM)
         a. In healthy horses, the post-ACTH cortisol concentration is expected to be 2–3 times the pre-sample's concentration.[2] However, four healthy horses had post-ACTH cortisol concentrations that ranged from 3.7 to 4.7 times the pre-ACTH concentrations.[9]
         b. In most but not all PDH horses, post-ACTH cortisol concentrations are > 3 times the pre-ACTH sample concentration.[9,27]
         c. Because of overlapping findings for healthy horses and horses with PDH, the ACTH stimulation test is not recommended as a diagnostic test for equine PDH.[9]
      3. Results expected for healthy and PDH horses if ACTH (cosyntropin) is given (100 IU IV)
         a. In healthy horses, the post-ACTH cortisol concentration is expected to be < 1.8 times the pre-sample's cortisol concentration.[28]
         b. In PDH horses, the post-ACTH cortisol concentration is expected to be > 1.8 times the pre-sample's cortisol concentration.

COMBINED DEXAMETHASONE SUPPRESSION/ADRENOCORTICOTROPHIC HORMONE (ACTH) STIMULATION TEST

I. 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.

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